INTERACTIONS OF MICROTUBULE-ASSOCIATED PROTEIN-2 WITH MICROTUBULES AND NEUROFILAMENTS

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ATP, adenosine triphosphate

cAMP, cyclic adenosine monophosphate

cDNA, complementary DNA

DEAE, diethylaminoethyl

GTP, guanosine triphosphate

HPLC, high pressure liquid chromatography

MAP, microtubule-associated protein

mRNA, messenger RNA

NF, neurofilament

pI, isoelectric point

PMSF, phenylmethanesulfonyl fluoride

SDS, sodium dodecyl sulfate

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Bovine brain microtubule-associated protein-2 (MAP-2) is a 280 kDa protein that binds to microtubules and neurofilaments. MAP-2 was fragmented by thrombin into a 240 kDa projection domain and a 28 kDa microtubule-binding domain. The 28 kDa cleavage fragment possessed a neurofilament binding site for the L subunit of neurofilaments. The thrombin cleavage site of MAP-2 was very similar to the chymotryptic cleavage site of the microtubule-associated protein tau. The 28 kDa microtubule-binding fragment was derived from the carboxyl terminus of the intact protein and the 240 kDa projection domain was from the amino terminus. The microtubule-binding fragment was very rich in lysine and arginine residues and its isoelectric point was approximately 10.0. The microtubule-binding fragment contained three octadecapeptide imperfect repeats located fifty residues from the thrombin cleavage site. These sequences were chemically synthesized to assay

for promotion of tubulin polymerization. Only the second promoted tubulin polymerization *in vitro*, yielding microtubules of normal morphology. The time course of peptide-induced microtubule assembly was similar to microtubule-protein *in vitro*. This octadecapeptide displaced MAP-1b from MAP-containing microtubules. The addition of the next three amino acids in the MAP-2 sequence to the carboxyl terminus of the peptide increased its ability to promote tubulin polymerization at lower concentrations and displaced all high-molecular-weight MAPs, MAP-1a,b and MAP-2a,b. This extended peptide displayed competitive binding with radiolabeled MAP-2 to taxol-stabilized MAP-free microtubules, suggesting the peptide bound to the same site on microtubules as MAP-2. The dissociation constant for MAP-2 binding was 3.4 μM in the absence of the extended peptide and 14 μM in the presence of 1.5 mM peptide. The estimated inhibition constant for the extended peptide is 0.5 mM, about 100 times lower than for the K_m of MAP-2. These observations suggested that the second repeated sequence of MAP-2 represents an important recognition site for MAP-2 binding to microtubules and that other structural features within MAP-2 may reinforce the strength of MAP-microtubule interactions.

CHAPTER 1
INTRODUCTION

An Overview of the Cytoskeleton

The cytoskeleton of eukaryotic cells is a dynamic organelle responsible for maintaining cell shape and rigidity, cell motility, and intracellular vesicle transport and trafficking. The cytoskeleton consists of three main types of filaments: microfilaments, intermediate filaments, and microtubules. Microfilaments are seven nanometers in diameter and are composed primarily of actin but also contain actin-binding proteins. Intermediate filaments are ten nanometers in diameter and vary in composition depending on the particular cell type. In brain tissue, the neuronal cell intermediate filaments are made of neurofilament proteins while in glial cells, the intermediate filaments are composed of glial fibrillary acidic protein. Microtubules are 24 nanometer diameter structures composed mainly of the heterodimer tubulin. In addition to tubulin, there are cell specific microtubule-associated proteins (MAPs) that bind to microtubules.

When specific cytoskeletal filaments are mixed *in vitro*, they interact with each other. If bovine spinal cord neurofilaments are mixed with bovine brain microtubules, the viscosity of the resulting solution increases greatly and a gel is produced (Runge et al., 1981). This is thought to be

a relevant and physiological interaction based on microscopical examination of neuronal axoplasm which shows neurofilaments and microtubules running parallel to each other in neurite processes. The work of Hirokawa and his coworkers over the years has demonstrated through microscopy techniques that projections exist between the filamentous structures (Hirokawa et al., 1985; Hirokawa 1982). These projections could be cross-bridges connecting the two types of filaments. Possible candidates for the cross-bridges are MAPs. Both microtubule-associated protein-2 (MAP-2) and tau proteins are MAPs and can bind to neurofilaments as well as microtubules (Letterier et al., 1982; Heimann et al., 1985; Miyata et al., 1986). It is the role of MAPs, specifically MAP-2 and its interactions with microtubules and neurofilaments, that is the focus of this thesis.

Microtubules

Microtubule Structure and Function

Microtubules are the most dynamic component of the cytoskeleton and exist in all eukaryotic cells except enucleated erythrocytes. The microtubule core is formed from alpha and beta tubulin heterodimers arranged into parallel rows, extending the length of the tubule, termed protofilaments. Isolated microtubules and those observed in sectioned cell specimens contain thirteen protofilaments, while most microtubules assembled *in vitro* possess fourteen protofilaments (McEwen and Edelstein, 1980). The tubulin subunits arrange themselves in a head-to-tail fashion along the protofilaments yielding the distinct polarity in

microtubules. This polarity was first observed by Rosenbaum and Child (1976) and Witman (1975), who demonstrated biased addition to microtubules in vitro. One end of the microtubule polymer displays an increased rate of addition compared to the other end. This characteristic polarity of microtubules can be determined by interactions with microtubule-binding proteins. One method is based on the interaction of dynein with assembled microtubules (Haimo et al., 1979; Haimo, 1982). Dynein is a large protein found in flagella of Tetrahymena that binds to microtubules and hydrolyzes adenosine triphosphate to produce the whip-like motion during movement. The main globular head of the dynein molecule tilts at an angle of 55° in the direction of the end of preferred growth of the microtubule. Another method to distinguish microtubule polarity takes advantage of special in vitro solution conditions that favor formation of microtubule walls decorated with additional protofilaments. The extra protofilaments align themselves into hook-shaped sheets and curve in one specific direction depending on the polarity; either clockwise or counterclockwise (Burton and Himes, 1978).

This intrinsic polarity of microtubules enables microtubules to perform vectorial functions. When intracellular vesicles travel along a microtubule in an axon, the vesicle generally moves in one direction. Rarely will a vesicle change its direction after starting its journey, and most vesicles move toward the cell body in a neuron (Hollenbeck and Bray, 1987). The polarity of a

microtubule is clearly a vital property for interactions with other cytoskeletal components as well as for their proper function. In a non-neuronal cell, microtubules are capped at their minus end near the centrioles and extend the plus end to the cell margin, yielding an overall radial polarity in the cell.

Some other interesting properties of microtubules are that they self-assemble in vitro in the presence of GTP at warm temperatures and that they depolymerize at cold temperatures or upon the addition of calcium ions. Each alpha/beta dimer binds two moles of GTP per mole tubulin, but each heterodimer has two types of nucleotide binding sites (Weisenberg et al., 1968; Berry and Shelanski, 1972). The beta subunit has an exchangeable nucleotide binding site that readily exchanges GDP for GTP. The alpha subunit has a nonexchangeable binding site that exchanges GDP for GTP very slowly. Guanosine triphosphate nucleotides are hydrolyzed only upon or after heterodimer incorporation into the polymeric tubule.

Both in vitro and in vivo microtubule dynamics have been monitored through the use of tubulin modified with a fluorescent tag. This tag can be a direct covalent attachment of a fluorochrome or by using an anti-tubulin antibody with a secondary antibody labeled with a fluorescent probe. In most cases examined the microtubules have been shown to be highly dynamic structures rapidly polymerizing and depolymerizing depending on the surrounding conditions (Mitchison and Kirschner, 1984; Kristofferson et

al., 1986; Sammak et al., 1987). In vitro, the addition of MAPs reduces microtubule dynamics (P.S. Yamauchi, personal communication).

Microtubules are essential for proper mitotic function. When the mitotic spindle poison colchicine is added to cells, mitotic arrest is seen; particularly at metaphase prior to microtubule depolymerization in anaphase which achieves chromosome movement toward the spindle poles. The chromosomes are connected to the microtubules through kinetochores which cap the microtubules at one end, while the opposite end is capped by the spindle pole or centriole. During interphase the centrioles seem to act as microtubule organizing centers and are located just outside the nucleus.

Some other important functions of microtubules are the maintenance of cell anisometry and promotion of cell shape changes. Nerve axons and retinal rod cells rely on the microtubule network to support their distinctive cell morphology, especially the neurite processes (Heidemann et al., 1986). Conversely, the activation of platelets and the action of polymorphonuclear leukocytes depend on changes in the microtubule-cytoskeleton (Malawista, 1986). Microtubules also provide the basic framework for the cell motility machinery as they are essential components of flagella and cilia.

Tubulin

The main component of microtubules is tubulin. This protein exists as a heterodimer of 100 kDa with each subunit possessing a molecular mass of approximately 50 kDa. There

is considerable homology between the α and ß subunits suggesting a common ancestral origin (Valenzuela et al., 1981). Both α and ß tubulin are very conserved across species lines suggesting that there are stringent requirements on the structure of tubulins over a reasonably great phylogenetic range (Cleveland and Sullivan, 1985). Both subunits contain a glutamate-rich carboxyl terminus that is thought to be responsible for MAP binding to microtubules (see Fig. 1-1). When the carboxyl termini of both subunits are proteolytically removed with subtilisin, the tubulin can self-assemble without MAP binding (Serrano et al., 1984). In fact, when these tubules are sedimented and the pellet fraction analyzed, the MAPs are found only in the supernatant fraction. The presence of high concentrations of salt (0.4 M -0.6 M) disrupts MAP binding to microtubules indicating the interaction of MAPs with microtubules is dependent on the ionic interactions. Thus the glutamate rich carboxyl termini are thought to be the binding sites for MAPs which in turn contain positively charged residues (Littauer et al., 1986).

Neurofilaments

Neurofilament Structure

Neurofilaments are one member of the intermediate filament protein family of which there are five main components: (1) acid keratins, (2) basic keratins, (3) glial fibrillary acidic protein, vimentin, peripherin, and desmin, (4) neurofilaments and alpha-internexin, and (5) lamins. Keratins are found in epithelial cells and their

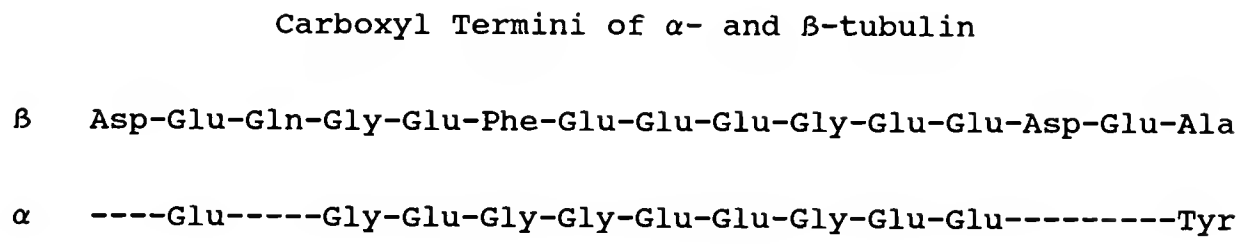


Fig. 1-1 Comparison of the carboxyl termini of both α- and ß-tubulin from chick brain (Valenzuela et al., 1981). Note the high glutamate content of both sequences.

derivatives such as skin and nails (Steinert and Roop, 1988). Desmin filaments are found mostly in muscle cells while vimentin filaments are located in mesenchymal cells (Steinert and Roop, 1988). Glial fibrillary acidic protein is the basic building block of glial filaments which are found in glial cells (Steinert and Roop, 1988). Peripherin is located in the neurons of the peripheral nervous system (Portier et al., 1984) while neurofilament proteins are found in most neuronal cells (Steinert and Roop, 1988). These filaments are composed of three subunits. The main core of the filament is made of the L subunit, a 70 kDa protein. The other two subunits are the M subunit and H subunit which are 150 kDa and 210 kDa respectively. Both of these subunits contain multiple phosphates on serine and threonine residues.

The neurofilament proteins have some common structural features with each other and all other intermediate filament proteins. Each has a 40 kDa conserved rod domain believed to be derived from a common ancestral gene (Weber et al., 1983). This common rod-shaped region is rich in alpha helical content and possesses a very conserved epitope that reacts with a mouse monoclonal antibody that recognizes all intermediate filament proteins (Pruss et al., 1981). This 40 kDa region has been conserved and is essential for intermediate filament assembly (Steinert et al., 1981; Geisler and Weber, 1981). Each intermediate filament protein contains hypervariable regions that flank the central rod region. These hypervariable regions form the

amino and carboxyl termini of the proteins also known as the head and tail regions respectively. It is the variation in these regions that distinguishes each individual intermediate filament protein. The difference between neurofilament proteins and other intermediate filament proteins like desmin and vimentin is the tail region. Normally the tail region is only approximately 5 kDa in mass but is over 55 kDa in the H subunit of neurofilaments and 50 kDa in the M subunit. It is the tail regions of the M and H subunits (see Fig. 1-2) that contain many of the phosphorylation sites found in these proteins (Julien and Mushynski, 1983). The tail region of the L subunit is considerably shorter than the M or H subunits and is rich in glutamate content which may be important in MAP binding similar to the glutamate rich carboxyl termini of alpha and beta tubulin. The tail regions are considerably less conserved across species lines.

Neurofilament Function

The neurofilament proteins are found mostly in axons as opposed to the cell body and dendrites. Once assembled into polymers, neurofilaments do not easily dissociate (Giesler and Weber, 1981; Moon et al., 1981). In fact, they are insoluble in aqueous buffers and their purification relies on solubilization with high concentrations of urea (Tokutake, 1984). Upon removal of the urea by dialysis, the filaments reassemble. Filament formation after dialysis from urea is not restricted to the neurofilament triplet (i.e. neurofilaments composed solely L, M, and H subunits),

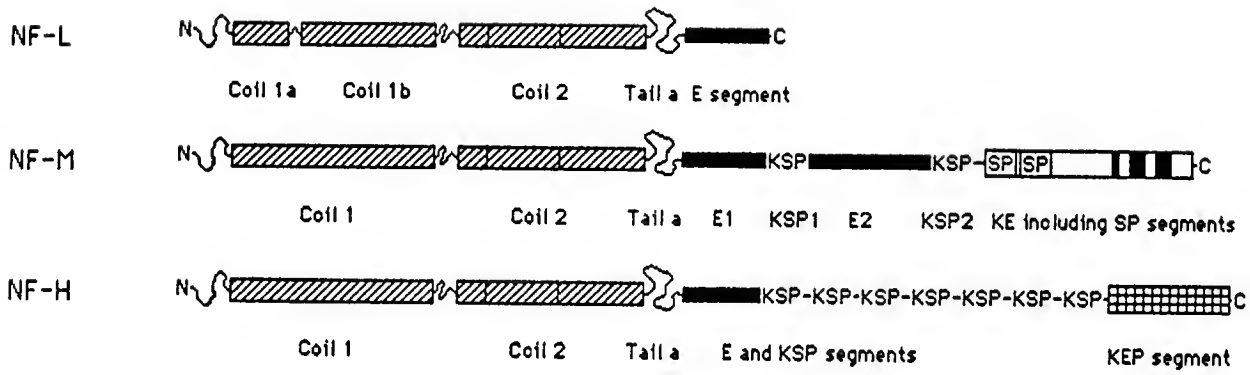


Fig. 1-2 Structural organization of the neurofilament triplet proteins. All three proteins contain coil-coil regions common to all intermediate filament proteins. Differences are seen in the amino terminal heads and carboxyl terminal tails. The phosphorylation sites in NF-M and NF-L are in the KSP-rich sequences.

but for each individual subunit when purified from the other two subunits can also be assembled by this method although the filaments are not as structurally intact for the M and H subunits (Tokutake et al., 1984). Unlike the case of facile microtubule assembly/disassembly, intermediate filament proteins form long-lived structures that provide the only known function of these proteins, namely stabilization and maintenance of the axon's structural integrity and caliber. Neurofilaments increase the size of an axon by adding to the volume occupied by the cytoskeleton (Lasek et al., 1983). The contribution to axonal diameter or caliber, has a direct effect on axonal function because the larger the axonal diameter, the faster the action potential travels down the axon. Also, this increase in size permits specialization of nerve cells because the largest axons have the ability to excite several target nerve cells at one time (Zucker, 1972). This can synchronize an entire population of cells to act coordinately as with muscle cells to produce movement. Axon size is very important to organisms which must coordinate large muscles to produce rapid and essential processes necessary to organismal survival. With a minimum number of junctions, axon size is very important and expression of the neurofilament genes can be crucial for proper organismal behavior and survival (Bullock and Horridge, 1965). Interestingly, some axons contain little neurofilament proteins but still function properly apparently as a result of increased microtubule content that

can replace neurofilaments in increasing the size of the axon (Morris and Lasek, 1984).

While neurofilaments are required architecturally for proper axon function, they are not required for such axonal dynamics as axonal transport which is dependent on microtubules. When an axon is disrupted by the neurotoxin ß,ß'-iminodipropionitrile, the axonal inner structure is rearranged with neurofilaments segregating to the outer regions of the axon diameter and microtubules and organelles segregating toward the center (Papasozomenos et al., 1981). This model system has been used to examine interactions with microtubules and neurofilaments where it has been shown that cross-links exist between microtubules and neurofilaments as well as within neurofilament networks themselves. An antibody specific for MAP-2 has been found to co-localize with both groups of filaments in iminodipropionitrile-treated neurons implicating MAP-2 as a cross-linker within and between these cytoskeletal systems (Papasozomenos et al., 1985).

Microtubule-Associated Proteins

Introduction

MAPs are a varied class of proteins that have been classified on the basis of their binding or modification of microtubules. When one considers the diversity of microtubule functions, it is not surprising that there exists a great number of proteins that regulate temporal, spatial, and metabolic controls of microtubule processes (see Table 1-1). The first attempt to characterize proteins

Table 1-1

Major classes of microtubule-associated proteins

Protein	Subspecies	Subunit mass (kDa)[a]	Primary source	Properties
MAP 1	1A,1B	350	brain	thermolabile;projection on microtubule
Light chains		28,30	brain	associated with MAP-1
MAP 1C	--	350	brain	cytoplasmic dynein
MAP 2	2A,2B	270	brain	thermostable;projection on microtubule; separable into projection (235 kDa) and binding (35 kDa) domains phosphorylated;binds calmodulin
Type II cAMP-dependent protein kinase		53,39		associated with MAP-2 projection domain
Tau	3-5	55-62	brain	thermostable;number of peptides depends on age and species; phosphorylated;binds calmodulin
MAP 3	--	180	brain	

Table 1-1 Continued

Protein	Subspecies	Subunit mass (kDa)[a]	Primary source	Properties
MAP 4;210-kDa HeLa MAP; 205-kDa Drosophila MAP	3-4	200-240 depending on species	cultured mammalian cells; mouse tissues (MAP 4); Drosophila (205-kDa)	thermostable
125-kDa MAP	--	125	cultured mammalian cells	
Chartins	--	69,72,80	cultured mammalian cells;primary neurons	thermolabile;phosphorylated
STOPs	--	140,72,56	brain	associated with cold-stable microtubules
Sea urchin MAPS	--	37,78,80 150,200,235	sea urchin eggs; sea urchin spindles	spindle localization
Kinesin	--	110 134	squid axoplasm sea urchin eggs	moves particles on microtubles
Dynamin	--	100	calf brain	microtubule-activated ATPase producing movement of microtubules, bundles microtubules

[a]Denatured mass of major polypeptides in each class as determined by SDS-PAGE.

associated with microtubule-networks was by Gibbons (1965) who demonstrated that dynein could be selectively released and then rebound to axonemes of Tetrahymena. Later, Weisenberg (1972) observed that microtubules can be assembled in vitro from crude brain extracts, and this observation led to the identification of proteins associated with polymerized microtubules. Through the use of repetitive cycles of temperature-dependent assembly and disassembly (Shelanski et al., 1973), quantitative MAPs or MAPs that bind to microtubules in a defined molar ratio were identified. The two most obvious classes of MAPs in this category were the high-molecular-weight MAPs, MAP-1 and MAP-2, and a family of polypeptides known as tau which has a molecular mass of 55 to 68 kDa.

Unfortunately, the use of temperature-dependent cycles has obscured the discovery of many less abundant, but potentially very significant MAPs. During the course of several temperature-dependent cycles much protein is lost, both tubulin and MAPs. Only appoximately fifteen percent of the microtubule-protein is yielded from bovine brain tissue after three cycles. Also the use of nucleotides, both adenosine- and guanosine triphosphate, increases the yield of microtubule-protein by increasing the amount of tubulin that polymerizes, but also influences MAP associations with microtubules. For instance, the inclusion of adenosine triphosphate in the first warming of the brain tissue extract releases MAP-1C, a cytoplasmic retrograde dynein, from the microtubule-lattice (Paschal et al., 1987b). This

protein was not characterized as a microtubule-associated protein for years because it was routinely discarded during the cycling of microtubule-protein.

One of the high-molecular-weight MAPs already mentioned is MAP-1 which is actually a family of polypeptides at appoximately 350 kDa. There are two closely spaced bands on denaturing polyacrylamide gels that are MAP-1a and MAP-1b. Even though they are similar in molecular weight they exhibit different monoclonal antibody reactivities and produce different peptide digest patterns. These proteins exhibit no preferential localization in brain tissue but their purification from white matter as a family takes advantage of the fact that other high-molecular-weight contaminants (i.e. MAP-2) are enriched in gray matter (Vallee, 1986). There is no available purification to date for separating the subspecies of this family. MAP-1C on the other hand has been purified to homogeneity and found to be a microtubule-activated ATPase that can translocate microtubules on glass slides and can translocate vesicles along microtubules in an ATP-dependent fashion (Shpetner et al., 1988). This very large protein has a native molecular mass of approximately 450 kDa and scanning transmission electron microscopy revealed that MAP-1C has a morphology and mass of a two-headed dynein (Vallee et al., 1988). Additionally, ultraviolet irradiation in the presence of vanadate cleaved the protein into two fragments of about the same size as those produced from flagellar dynein (Paschal et al., 1987a).

Another microtubule-activated ATPase that can translocate microtubules as well as intracellular vesicles is kinesin. Kinesin was first discovered during studies of organelle transport in the giant squid axon, where microtubules serve as tracks for the movements of organelles (Vale et al., 1985a). Kinesin isolated from squid axoplasm can induce movement of carboxylated latex beads along purified microtubules or gliding of microtubules on glass in the presence of ATP (Vale et al., 1985b). The direction of movement along the microtubules was anterograde or towards the nerve terminal in an axon. This is exactly the opposite direction of movement as MAP-1C (Paschal and Vallee, 1987). Kinesin has since been found in a variety of organisms and cell types from mammalian brain tissue (Brady, 1985) to Drosophila melanogaster (Saxton et al., 1988). This wide distribution of kinesin suggests it may be involved in a variety of microtubule-based motility systems in different cell types. Immunolocalization studies in sea urchin eggs and some mammalian cultured cells have demonstrated kinesin is located in the mitotic spindle suggesting a role for kinesin in mitosis (Scholey et al., 1985).

Kinesin is composed of two heavy chains of 120-124 kDa and two light chains of 62-64 kDa and is a highly elongated molecule with an axial ratio of appoximately 20:1 (Bloom et al., 1988). Rotary shadowing of kinesin shows it is a rod-shaped molecule approximately 80 nanometers long. One end of each kinesin molecule contains a pair of globular domains while the opposite end is fan shaped (Hirokawa et al.,

1989). Monoclonal antibodies against the heavy chains stain the globular structures while antibodies versus the light chains stain the fan-shaped end (Hirokawa et al., 1989). A 60 kDa amino terminal section of the heavy chain corresponds to the globular head region and contains the nucleotide-dependent microtubule binding activitity and is thought to the motor domain (Yang et al., 1989).

Among the lesser known MAPs is the MAP-4 class which consists of a series of MAPs with molecular masses around 200-240 kDa depending on the species. It is found in almost all cultured mammalian cells, in mouse tissues, and in Drosophila melanogaster. MAP-4 shares the property of thermostablity with MAP-2, a feature that is rare in proteins of their size. The overall structure and binding to microtubules of a few MAP-4 types are just starting to be analyzed. Another group of not very well characterized MAPs are STOPs which confer cold-stability on microtubules (Margolis and Rauch, 1981). Normally, microtubules depolymerize when exposed to cold temperatures; in the presence of STOPs, the microtubules are stable. These tubules can be depolymerized by calmodulin and low calcium concentrations, and it has been shown that these proteins are all retained on a calmodulin affinity resin (Job et al., 1982).

Tau Proteins

Among the best characterized MAPs to date are the tau proteins, a family of closely related polypeptides. On denaturing polyacrylamide gels the proteins exhibit

molecular masses of 55 to 70 kDa with usually four or five distinct bands appearing. Tau is a phosphoprotein with serines and threonines primarily phosphorylated. It is located in neuronal tissues and restricted to axons of neurons. Tau has been found in paired helical filaments and in plaques from Alzheimer brain patients (Goedert et al., 1988). It has the remarkable properties of being soluble in 2.5% (v/v) perchloric acid and being insoluble in 25% (v/v) glycerol (Lindwall and Cole, 1984). Tau also is heat-stable, and its purification takes advantage of this property.

The source of heterogeneity in tau was unclear until recently when it has been demonstrated that the different tau polypeptides are the result of alternative splicing from one mRNA transcript (Himmler, 1989). By the selective splicing of specific exons from the transcript, a specific tau translation product is synthesized. This work was performed with bovine brain mRNA. The first tau sequence to be identified was the murine system by Lee et al. (1988) who showed two tau transcripts were made <u>in vitro</u> with differing carboxyl termini. An interesting structural feature of the transcripts noticed in the murine tau investigation was the presence of three imperfect octadecapeptide repeats in the center of the molecule. Each repeated sequence ended with a proline followed by three glycines. All the repeats contained serines and threonines, and they were rich in the basic amino acids arginine and lysine. These investigators

hypothesized the repeats were important for tau binding to microtubules.

Another group of investigators showed a proteolytic fragment of tau could bind to microtubules (Aizawa et al., 1988). A 14 kDa chymotryptic fragment present in each bovine tau polypeptide was found to bind to microtubules. The amino terminus of this fragment was determined by Edman sequencing which localized where the fragment was derived from after comparison with the murine cDNA predicted sequence. This proteolytic fragment contained two of the three repeats believed to be involved in microtubule binding. This was the first indication that not all the repeats were necessary for tau binding to microtubules. An interesting feature of bovine tau protein not elucidated by the protein chemistry work of Aizawa et al. (1988) was shown by the cDNA sequencing work of Himmler et al. (1989). They determined that all the bovine forms of the tau polypeptides contain four repeats rather than three as seen in the murine forms. It is unclear what the significance of having four repeats versus three repeats is although tighter or better binding could be the result.

Some interesting work with the repeats of tau has been done <u>in vitro</u>. Ennulat et al. (1989) has shown that synthetic peptides corresponding to the first and second repeats of murine tau protein can promote the polymerization of tubulin. The third repeat has also been tested but failed to promote tubulin polymerization into microtubules. Himmler et al. (1989) also has demonstrated that a

polypeptide consisting of the four repeats can cosediment with taxol-stabilized microtubules and he demonstrated that just two repeats fused together also possessed this ability. The importance of the cosedimentation data cannot be overlooked, but the work of Ennulat et al., (1989) is more significant because this was the first demonstration of a synthetic peptide of a microtubule-associated protein performing the same function as a MAP.

Interestingly, Aizawa et al. (1989) found a sequence that is similar to one of the repeats of tau in the 190 kDa adrenal gland-specific microtubule-associated protein. This sequence could also promote the polymerization of tubulin in vitro. An emerging theme in microtubule cytoskeletal research is that a group of similar sequences in some MAPs are responsible for promoting MAP-tubule interactions.

Microtubule-Associated Protein-2

Microtubule-associated protein-2 (MAP-2) is a very large protein specific for neuronal tissue and restricted to dendrites. Its molecular mass on denaturing gels is approximately 280 kDa but the predicted mass from its cDNA is only about 200 kDa (Lewis et al., 1988). MAP-2 is very similar to tau in that it is heat-stable, a phosphoprotein, and the murine form contains a trio of imperfect octadecapeptide repeats. MAP-2 also shows heterogeneity on denaturing gels splitting into two high-molecular-weight forms, MAP-2a and MAP-2b. It is unknown what the cause of this heterogeneity is. The only known function of MAP-2 is to polymerize tubulin into microtubules.

MAP-2 can be phosphorylated by a variety of kinases. The cAMP-dependent protein kinase, calmodulin dependent protein kinase, calcium/phospholipid-dependent protein kinase, and protein kinase C have all been shown to use MAP-2 as a substrate (Goldenring et al., 1985; Tsuyama et al., 1986; Akiyama et al., 1986). MAP-2 can also be phosphorylated by non-neuronal specific kinases such as the insulin receptor kinase and an epidermal growth factor stimulated kinase (Kadowaki et al., 1985; Hoshi et al., 1988). When MAP-2 is isolated by standard cycling procedures from brains it contains about 10 moles phosphate per mole of MAP-2 (Tsuyama et al., 1986). About ten more phosphates can be added with exogenous cAMP-dependent protein kinase. Rat brain MAP-2, isolated immediately following rapid <u>in vivo</u> heat-treatment, contains approximately 46 moles of phosphate per mole of MAP-2 (Tsuyama et al., 1987). Such microwave treatment reduces the activity of phosphoprotein phosphatases. The phosphorylation state of MAP-2 has a direct influence on its function. The more phosphorylated the protein is, the less its affinity for tubulin is and the ability to promote polymerization is reduced (Murthy and Flavin, 1983).

MAP-2 has very little organized secondary structure. Circular dichroic measurements of MAP-2 revealed it contained very little alpha-helix or beta-sheet (Hernandez et al., 1986). This same study determined that MAP-2 possessed a highly elongated structure as analyzed by gel filtration chromatography and analytical

ultracentrifugation. In addition, the predicted structure from the cDNA studies of Lewis et al., (1988) also revealed little organized secondary structure.

Surprisingly, when MAP-2 is digested with various endoproteases, only two major fragments are usually produced: the first, a small fragment approximately 28-36 kDa in mass and the second, a large 240 kDa fragment. The small fragment contains the microtubule-binding site of MAP-2 (Vallee, 1980; Flynn et al., 1987), and the large fragment is known as the projection domain because it is seen in electron micrographs before protease treatment protruding from the microtubule wall but is absent after protease digestion (Vallee and Borisy, 1978). This limited digest pattern with several proteases suggests some higher order of structure. A completely random structure would give greater heterogeniety in protease digests. If trypsin or chymotrypsin are employed, the digest patterns are more complex but the microtubule-binding products are 34-36 kDa.

The projection domain contains a majority of the phosphorylation sites. The significance of these phosphorylation sites in the projection domain remains unclear. One known function of the projection domain is that it contains a binding site for the regulatory subunit of cAMP-dependent protein kinase (Vallee, 1986). This suggests that MAP-2 may be associated with a protein kinase. This kinase could phosphorylate the microtubule-binding domain thereby modulating the affinity of MAP-2 for microtubules.

The entire cDNA structure of murine MAP-2 recently was reported by Lewis et al. (1988), and it showed that MAP-2 possessed three imperfect octadecapeptide repeats similar to murine tau. Secondary structure predictions from the cDNA confirmed the findings of Hernandez et al. (1986) showing MAP-2 contained little alpha-helical content or beta-pleated sheet content. A synthetic polypeptide of 100 amino acids of MAP-2 sequence containing the first and second repeats was shown to bind to MAP-stabilized microtubules and could cycle with these microtubules (Lewis et al., 1988). This report revealed extensive homology of MAP-2 with tau especially in the carboxyl termini of both proteins as depicted in Fig. 1-3. It will be interesting to know the bovine MAP-2 sequence to see if it contains three octadecapeptide repeats like murine MAP-2 or four repeats as in the bovine tau proteins.

Recently a third form of MAP-2 has been found in addition to MAP-2a and b (Garner et al., 1988). This protein is a 70 kDa heat-stable MAP that cross-reacts with MAP-2 antibodies but is only expressed in neonatal and juvenile rats. Northern blots of different developmental stages in rat show a 6 kilobase mRNA in neonatal brain tissue and a 9 kilobase mRNA in adult brain tissue when probed with a MAP-2 specific cDNA (Garner and Matus, 1988). When this 70 kDa protein, now termed MAP-2c, was sequenced, the sequence was the same as MAP-2a and b except for a 1,372 amino acid deletion corresponding to the central section of the adult form (Papandrikopoulou et al., 1989). This was

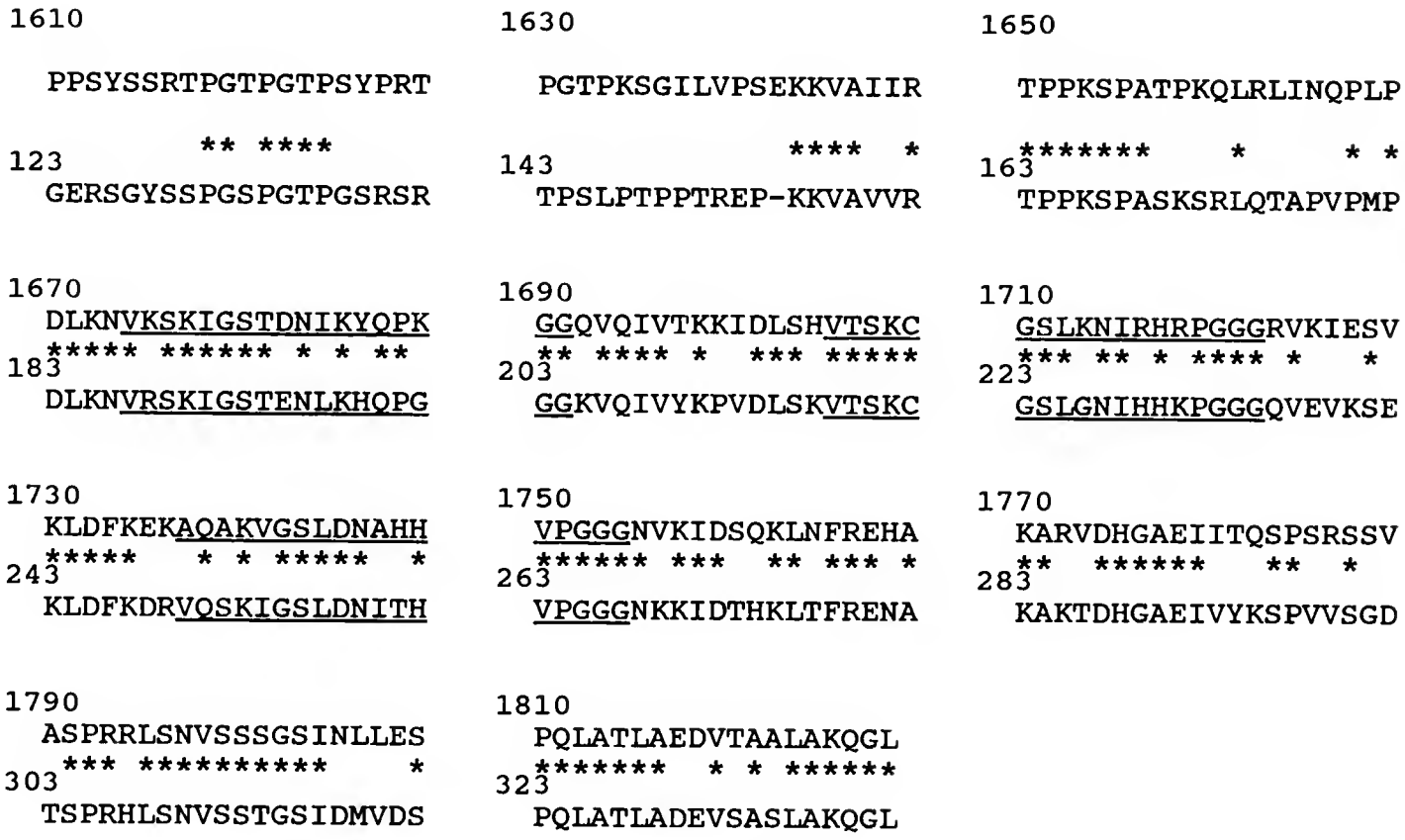


Fig. 1-3 Comparison of the carboxyl termini of murine MAP-2 (upper sequence) and murine tau protein (lower sequence). The asterisks indicate an exact match and the underlined sequences refer to the imperfect octadecapeptide repeats. The hyphen at position 155 of tau represents a space created to allow for better alignment of the sequences.

due to alternative splicing of the mRNA from one gene of MAP-2. The protein still contained the carboxyl terminus ofadult MAP-2 with the triad of imperfect repeats. It is currently unclear what the significance of the embryonic and adult forms is, although a reduced degree of cytoskeletal cross-linking during axonal and dendritic growth with the embryonic form is a possibility (Papandrikopoulou et al., 1989).

Altogether, the findings on the structural organization of MAP-2 can be depicted as shown in Fig. 1-4. This figure shows the section spliced out of mature MAP-2 creating the MAP-2c form. The binding site for the regulatory subunit of cAMP-dependent protein kinase is located at the amino terminus and the proposed microtubule-bundling domain is located at the extreme carboxyl terminus of the molecule. When this bundling sequence is removed, MAP-2 loses its ability to cause microtubule bundling (Lewis et al., 1989). This sequence contains homology to leucine zipper proteins and can be functionally replaced by these leucine zippers such as the one contained in GCN4. It has been hypothesized that the bundling domain at the carboxyl terminus of MAP-2 interacts with another MAP-2 carboxyl terminus causing the microtubules to bundle.

Proposal

The aim of this study was to determine how MAP-2 interacted with two different cytoskeletal components, neurofilaments and microtubules. This was to be accomplished through the use of proteolytic digests to

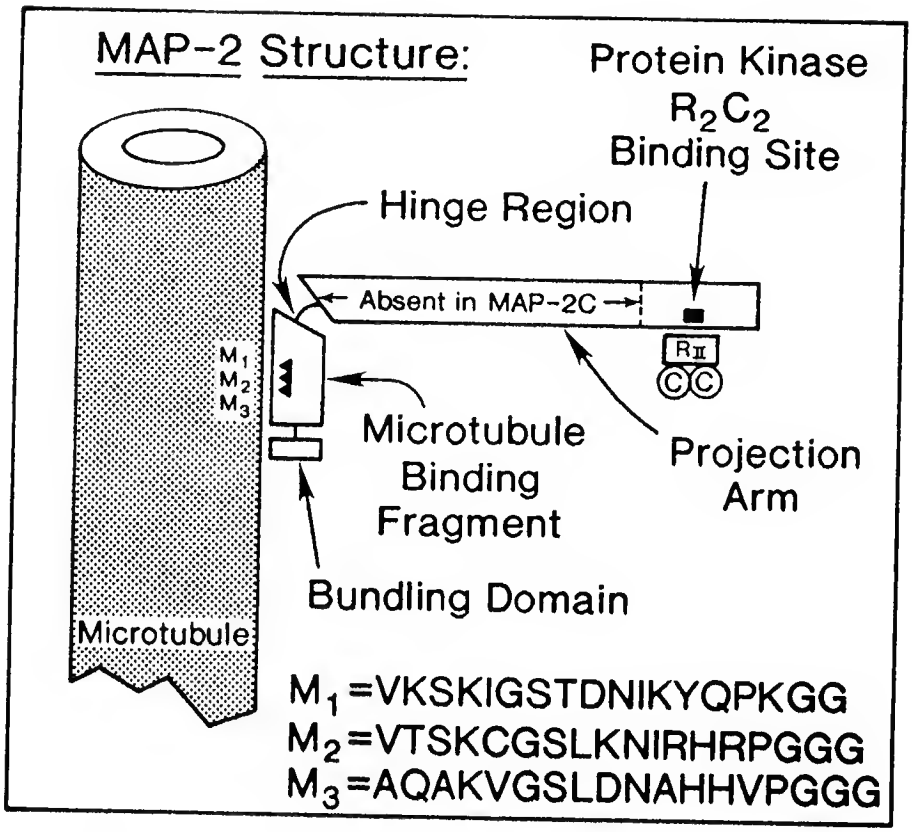


Fig. 1-4 Summary of MAP-2 structure in relation to a microtubule. The amino terminus of MAP-2 contains a binding site for the regulatory (R) subunit of cAMP-dependent protein kinase. The carboxyl terminus contains the imperfect repeats as well as a proposed bundling sequence. The hinge region contains a protease sensitive site.

determine which fragment(s) of MAP-2 could interact with the different polymers. Once the fragment was determined, a structural characterization of its biochemical properties was to be carried out. This included a determination of its isoelectric point, amino acid composition, and amino terminal analysis. With the availability of the entire sequence of murine MAP-2, internal sequences responsible for promoting tubulin polymerization *in vitro* were to be identified. In addition to tubulin polymerization, MAP-2 sequences responsible for displacement of high-molecular-weight MAPs from microtubules were to be identified.

CHAPTER 2
INTERACTIONS OF MAP-2 WITH TUBULIN AND NF-L

Introduction

Neuronal cytoplasm is highly organized, and both microtubules and neurofilaments run parallel with respect to the axon's longitudinal axis in a manner suggesting microtubule-to-neurofilament cross-linking (Wuerker and Palay, 1969; Ellisman and Porter, 1980; Hirokawa, 1982). *In vitro* observations indicate that microtubules interact with neurofilaments, and MAPs can enhance the attainment of high solution viscosity and/or gelation (Runge et al., 1981; Aamodt and Williams, 1984b; Minami and Sakai, 1983; Letterier et al., 1982). Aamodt and Williams (1984a) used falling-ball viscosmetry to demonstrate the occurrence of an optimal MAP level in plots of viscosity/gelation versus MAP concentration; they likened this MAP concentration profile to that of bivalent antibody cross-linking in immunoprecipitin formation. Previous studies have traced this apparently optimal MAP profile to the presence of endogenous GTPase activity, and the inhibition of cross-linking/gelation at high MAPs can be eliminated with a GTP-regenerating system (Flynn and Purich, 1987). The requirement of GTP is to maintain microtubule stability as microtubules will disassemble after all the GTP is converted to GDP. Nonetheless, high-molecular-weight MAPs do bind to

microtubules (Kim et al., 1979; Vallee, 1982; Purich and Kristofferson, 1984) and neurofilaments (Runge et al., 1981; Aamodt and Williams, 1984b; Minami and Sakai, 1983; Letterier et al., 1982), and some interactions of the 280 kDa neuronal MAP-2 have been explored by limited proteolytic fragmentation. Tubule binding is restricted to a 34-36 kDa tryptic or chymotryptic fragment of MAP-2, and the remaining 240 kDa component corresponds to the lateral projections observed in electron micrographs of microtubules decorated with MAP-2 (Kim et al., 1979; Vallee and Borisy, 1977).

During the course of studies on neurofilament-microtubule-MAP-2 interactions I sought to localize the site(s) of neurofilament binding with respect to the tubule-binding and -projection domains of MAP-2. A thrombin cleavage technique was developed to obtain these MAP-2 fragments in higher yields than that obtained with trypsin or chymotrypsin. Interestingly, a 28 kDa tubule-binding domain was found to contain a neurofilament-binding site. My studies also suggest that this binding interaction has considerable ionic character, as suggested by isoelectric point determinations of the MAP-2 fragment.

Materials and Methods

Materials

Bovine thrombin (catalog number, T 4648) and the catalytic subunit of cAMP-dependent protein kinase were purchased from Sigma. Ultrapure ammonium sulfate and urea were purchased from Schwarz-Mann, and carboxymethyl Sephadex from Calbiochem. [^{32}P]ATP (specific activity > 7000

Curies/mmol) was an ICN product, and Ampholines were obtained from LKB. Assembly buffer for preparation of microtubule-protein contained 0.1 M piperazine-N, N'-bis[2-ethanesulfonic acid], 1 mM ethyleneglycol-bis[ß-aminoethyl ether]-N,N,N',N'-tetracetic acid, and 1 mM magnesium sulfate.

Preparation of proteins

Bovine brain microtubule-protein was prepared by the procedure of Shelanski et al., (1973). Neurofilaments were prepared from fresh bovine spinal cord by the method of Delacourte et al., (1980) as modified by Letterier et al., (1982). Neurofilament triplet protein was prepared as described by Tokutake et al., (1983), and the NF-L subunit of neurofilaments was purified according to the method of Geisler and Weber (1981). Tubulin was separated from MAPs by the phosphocellulose method of Weingarten et al., (1975).

MAP-2 preparation and phosphorylation

MAP-2 was purified by the method of Herzog and Weber (1978), concentrated by ammonium sulfate precipitation, and phosphorylated by the catalytic subunit of cAMP-dependent protein kinase prior to gel filtration chromatography. Typically 500 units of kinase was dissolved in 0.025 ml dithiothreitol (50 mg/ml), incubated at room temperature for 10 minutes, and used immediately with 1.5 millicuries [^{32}P]ATP, 0.02 mM unlabeled ATP, for 30 minutes at 37°C in the presence of approximately 40 mg heat-stable MAPs. The MAPs were separated on a BioGel A-1.5M column with the MAP-2 fractions pooled and concentrated in a dialysis bag

against dry carboxymethyl Sephadex at 4°C. This purified [^{32}P]MAP-2 was clarified by centrifugation at 130,000 x g for 25 minutes in a Beckman Airfuge prior to digestion and incubations with cytoskeletal proteins.

Digestion of MAP-2 with thrombin

Purified and radiolabeled MAP-2 (50,000 CPM/μg) was incubated at 0.4 mg/ml with 4 units/ml thrombin. To determine the optimum time of digestion, aliquots were taken at 5 minute intervals from 0 to 30 minutes. Once an optimum time of 30 minutes was determined, all digestions of MAP-2 prior to incubation with cytoskeletal proteins were conducted for 30 minutes and quenched by the addition of 1 mM phenylmethylsulfonyl fluoride and incubation on ice.

Sedimentation of polymerized protein

Thrombin-digested or undigested MAP-2 was incubated with neurofilaments for 10 minutes at 4°C, or microtubules for 30 minutes at 37°C. The incubations were then layered over 20% (w/v) sucrose in assembly buffer and centrifuged at 130,000 x g for 20 minutes. Supernatant fractions were discarded and the pellet fractions were washed with 1 mg/ml bovine serum albumin and 0.1% (v/v) Triton X-100 and resuspended in 8 M urea. Aliquots of both supernatant and pellet fractions were analyzed for radioactivity by liquid scintillation spectrophotometry, and an equal number of counts were loaded on 7-17% (w/v) polyacrylamide gels.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate gel electrophoresis was carried out as described by Laemmli (1970), and nonequilibrium pH

gel electrophoresis (NEPHGE) was performed by the method of Roberts et al., (1984). For the NEPHGE gels radiolabeled MAP-2 was digested with thrombin and the digestion was quenched by the addition of an equal volume of 9.3 M urea, 0.5% (w/v) dithiothreitol and 2% (v/v) Nonidet NP-40 in 5 mM potassium carbonate. The digests were loaded onto "NEPHGE" tube gels with 1% (v/v) Ampholine 9-11 and 2% (v/v) Ampholine 3.5-10, and run for 2000 volt-hours. Gels were stained with coomassie blue, destained, dried under vacuum, and exposed to Kodak X-AR5 film at -80°C.

Results

Binding to microtubules and neurofilaments

In view of the high molecular weight of MAP-2, proteolytic fragmentation by trypsin or chymotrypsin has proven to be useful in defining the MAP-2 domain(s) interacting with other cytoskeletal components (Olmsted, 1986). Vallee (1980) first demonstrated that MAP-2 can be fragmented into 35 and 240 kDa components by chymotrypsin or trypsin. The smaller fragment contains the microtubule-binding domain, and the larger is designated as the projection-arm domain. While these protease cleavage products have been very useful in many investigations of microtubule self-assembly, chymotryptic and tryptic cleavage do not yield stable limit polypeptides, and the stability of such proteolytic fragments is quite limited, leading to the loss of the initially cleaved domains and the ability of these fragments to stimulate microtubule assembly. In a survey of the action of other proteases, I observed that

thrombin, an arginine-specific serine protease, predominantly yielded MAP-2 fragments of 28 kDa and 240 kDa. MAP-2 cleavage can be readily assessed by SDS gel electrophoresis of [^{32}P]MAP-2 because this protein is extensively phosphorylated (Theurkauf and Vallee, 1983). As seen in Fig. 2-1 this 28 kDa fragment is very stable and very resistant to further digestion over the time points shown. Indeed, phenylmethanesulfonyl fluoride at 1.0 mM final concentration blocked any further degradation over a five to seven day period at 4°C.

This development has allowed for probing with much greater ease the interactions of MAP-2 fragments with neurofilaments and microtubules. The basic approach is to determine which proteins or protein fragments cosediment with assembled microtubules or neurofilaments using ultracentrifugation and subsequent electrophoretic analysis. First MAP-2 was enzymatically phosphorylated with the cAMP-dependent protein kinase to a level of 1 mole of added phosphate per mole of MAP-2 based on the conditions of Letterier et al., (1982). After purification of the radiolabeled protein by gel filtration chromatography, the MAP-2 was concentrated, and aliquots were digested with thrombin. Next, the thrombin-digested fragments (indicated by the plus sign) or undigested MAP-2 (indicated by the minus sign) were incubated with microtubules or neurofilaments under the conditions listed in Fig. 2-2. The polymerized and unpolymerized cytoskeletal proteins were separated into pellet [p] and supernatant [s] fractions by

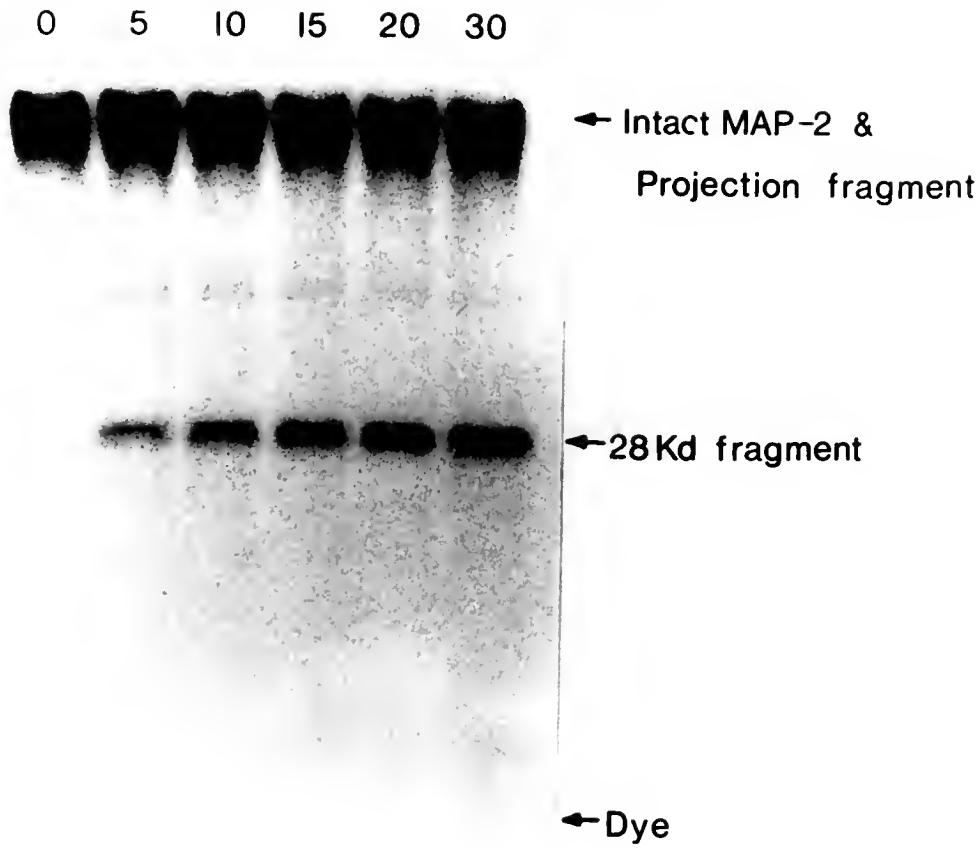


Fig. 2-1 Thrombin digestion of radiolabeled MAP-2. [^{32}P]MAP-2 was incubated at 37°C with 4 units/ml thrombin for the indicated time in minutes. The digestion was quenched by heating at 100°C for 5 minutes in the presence of sodium dodecyl sulfate and the products resolved on a 15% (w/v) polyacrylamide gel. The gel was then dried under vacuum and expose to Kodak X-AR 5 film.

ultracentrifugation. Because the projection and tubule-binding domains do not contain identical phosphorylation sites, a constant total amount of radioactivity was applied for each electrophoretic sample. Lanes 1-4 of the coomassie stained gel in Fig. 2-2 and the corresponding lanes of the autoradiogram in Fig. 2-3 demonstrate that only the 28 kDa thrombin-produced fragment of MAP-2 binds to neurofilaments. The next four lanes in both Fig. 2-2 and Fig. 2-3 demonstrate that this thrombin fragment behaves as the so-called microtubule-binding domain of MAP-2 as it binds to 1 mg/ml of taxol-stabilized microtubules composed solely of tubulin. In the absence of neurofilaments or microtubules the 28 kDa fragment remained in the supernatant fraction even after ultracentrifugation as seen in lanes 9-12 of Fig. 2-3. Indeed, the entire pellet fraction was used for the electrophoretic analysis in lanes 10 and 12 of Fig. 2-3, and virtually no high-molecular-weight or fragmented MAP-2 cosedimented without neurofilaments present. These observations verified that the fragment is only sedimentable as a result of interactions with either neurofilaments or microtubules.

Next, I sought to determine the neurofilament protein(s) interacting with MAP-2 or the 28 kDa fragment. Earlier work by Miyata et al., (1986) and Heimann et al., (1985) demonstrated MAP binding to the low-molecular-weight subunit of neurofilaments. A second series of binding assays were conducted and Fig. 2-4 shows the coomassie blue staining pattern and Fig. 2-5 shows the corresponding

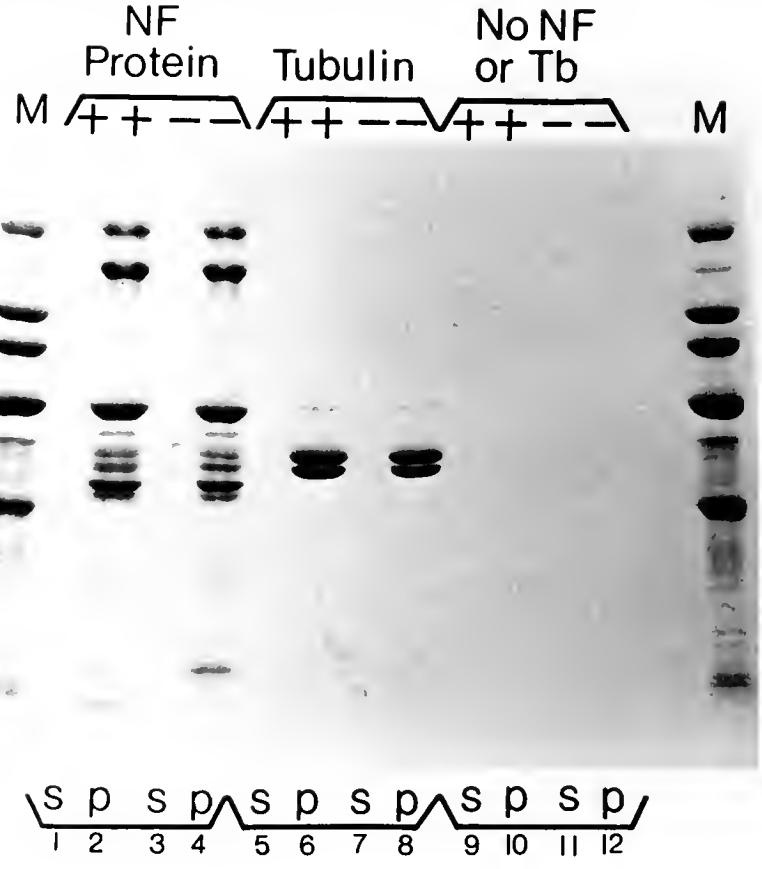


Fig. 2-2 Binding of MAP-2 or MAP-2 fragments to neurofilaments or tubulin. A coomassie blue stained gradient 7-17% (w/v) polyacrylamide gel is shown. Radiolabeled MAP-2 was incubated with (+) or without (-) thrombin as described in Fig. 2-1, quenched with 1 mM phenylmethylsulfonyl fluoride and incubated with 2 mg/ml neurofilament protein or 1 mg/ml taxol-stabilized tubulin with 1 mM guanosine triphosphate. Samples were then handled as described in "Methods". M refers to molecular weight markers, s to supernatant fraction, and p to pellet fraction.

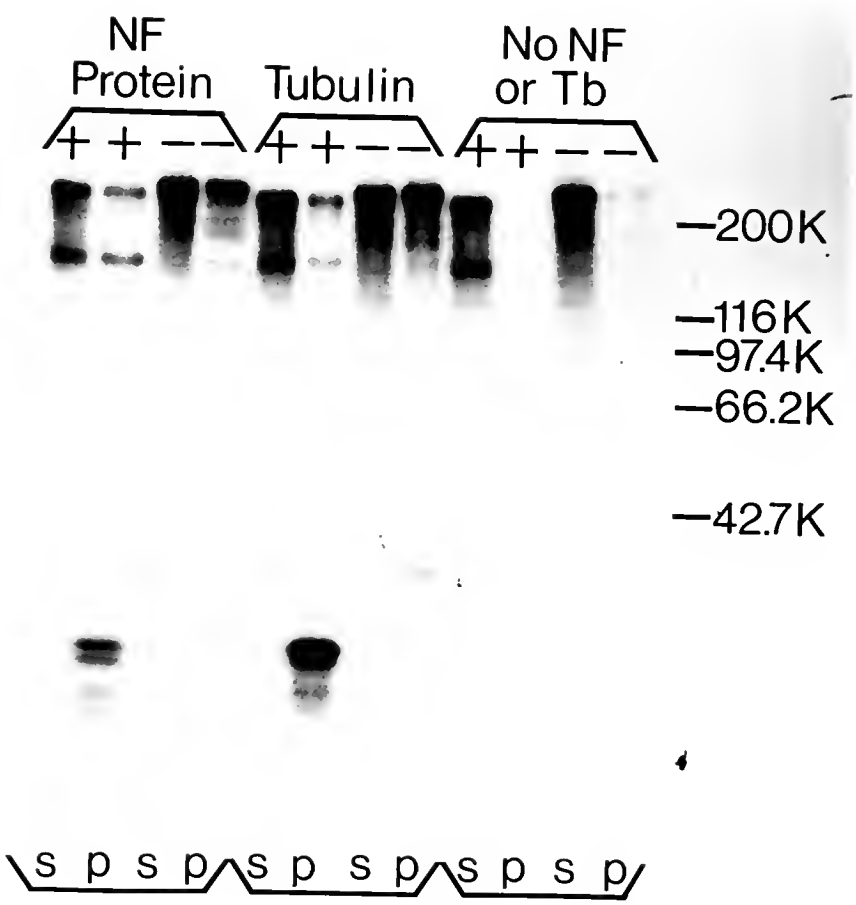


Fig. 2-3 Autoradiogram of MAP-2 and MAP-2 fragment binding to cytoskeletal protein. The coomassie blue stained polyacrylamide gel shown in Fig. 2-2 was soaked in 25% (v/v) glycerol for 30 minutes after destaining and dried under vacuum. The dried gel was exposed to x-ray film for 4 hours without intensifying screens.

autoradiogram. Lanes 3, 4 and 7, 8 of Fig. 2-5 verified that uncleaved MAP-2 binds to the neurofilament triplet and to filaments composed solely of L subunit. The data shown in lanes 1, 2 and 5, 6 of Fig. 2-5 extend the earlier observations by clearly demonstrating the binding of the 28 kDa fragment to the neurofilament triplet and L component of neurofilaments. It should be noted that only trace levels of tubulin are evident in the 50-55 kDa molecular weight range in the gels shown in Fig. 2-2. Much higher levels of tubulin are required for binding of MAP-2 to assembled tubules. This suggests that binding depends on presence of neurofilaments and does not require tubulin or assembled microtubules for binding.

Determination of the isoelectric point of the 28 kDa fragment

Tubulin and neurofilament proteins contain negatively charged regions that may be important in MAP binding (Olmsted, 1986). The data presented in the preceding section demonstrate that the 28 kDa fragment of MAP-2 binds to both cytoskeletal organelles. Accordingly, I tried to use conventional isoelectric focusing to determine the isoelectric point of the radiolabeled fragment. This consistently failed at all ranges of ampholytes, and the fragment never migrated into the first dimension of a two dimensional gel. This is indicative of very basic proteins. In order to estimate the isoelectric point of very basic neurofilament subunit proteins the Nonequilibrium pH gel electrophoresis technique is used (Roberts et al., 1984). When this technique was tried, the fragment readily migrated

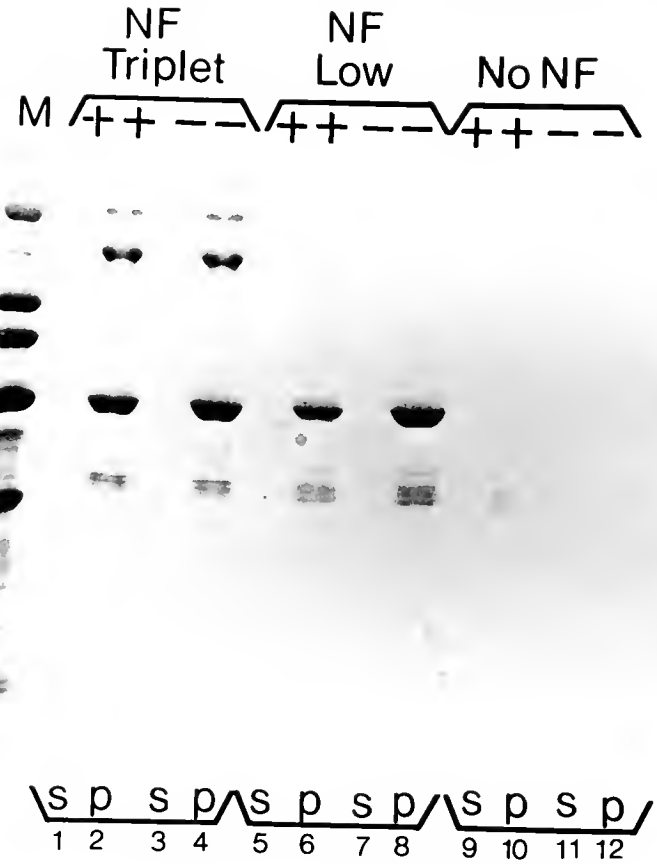


Fig. 2-4 MAP-2 binding to purified neurofilament triplet protein or the L subunit of neurofilaments. A coomassie blue stained gradient 7-17% (w/v) polyacrylamide gel is shown. Radiolabeled MAP-2 was incubated with thrombin as described in Fig. 2-2 and in "Methods", and incubated with either 2.2 mg/ml neurofilament triplet or 1.1 mg/ml L subunit at 4°C for 10 minutes. Nomenclature is the same as in Fig. 2-2.

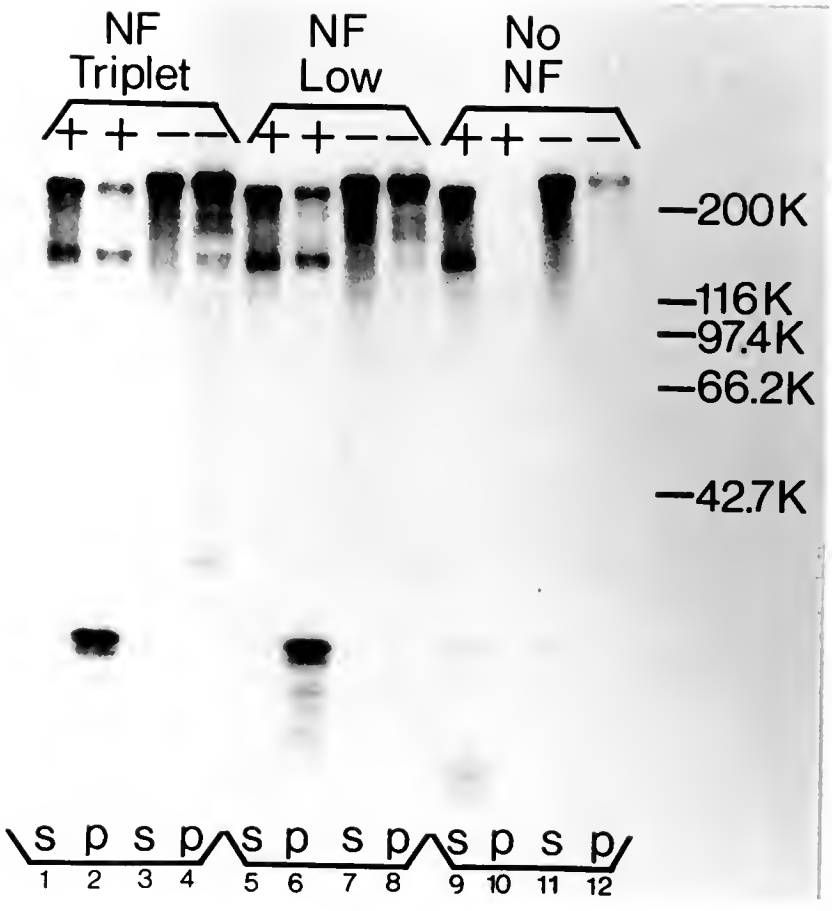


Fig. 2-5 Autoradiogram of MAP-2 and MAP-2 fragment binding to Neurofilament triplet protein and L subunit. The coomassie blue gel shown in Fig. 2-4 was soaked in 25% (v/v) glycerol for 30 minutes after destaining and dried under vacuum. The dried gel was exposed to x-ray film for 4 hours without intensifying screens.

into the first dimension of the gel as seen in Fig. 2-6, and it migrated between two very basic isoelectric point markers, ribonuclease (pI=9.3) and lysozyme (pI=10.5-11.0). The thick arrow denotes the position of the 28 kDa fragment in the gel. From this migration pattern, the isoelectric point of the 28 kDa fragment of MAP-2 was estimated to be approximately 10. The migration of the trace-labeled fragment in both dimensions also indicated that the phosphorylation conditions do not lead to significant heterogeneity in overall ionic charge or molecular weight. Furthermore, the dephosphorylated form will necessarily display an even higher isoelectric point. The large smear at the top right side of the gel is the projection domain and further breakdown products of this domain. These fragments are acidic and do not readily migrate into the gel.

Discussion

The findings presented in this chapter indicate that neurofilaments, a specific class of intermediate filaments, interact with MAP-2 in the region corresponding to the 28 kDa microtubule-binding domain. In earlier work with actin, Sattilaro (1986) reached a similar conclusion about the binding of chymotryptic fragments of MAP-2 to polymerized actin. Likwise, this same fragment of MAP-2 constitutes the microtubule-binding domain (Vallee, 1980). Thus, all three major cytoskeletal classes (i.e. microfilaments, intermediate filaments, and microtubules) interact with a common structural region of MAP-2. In view of our estimated

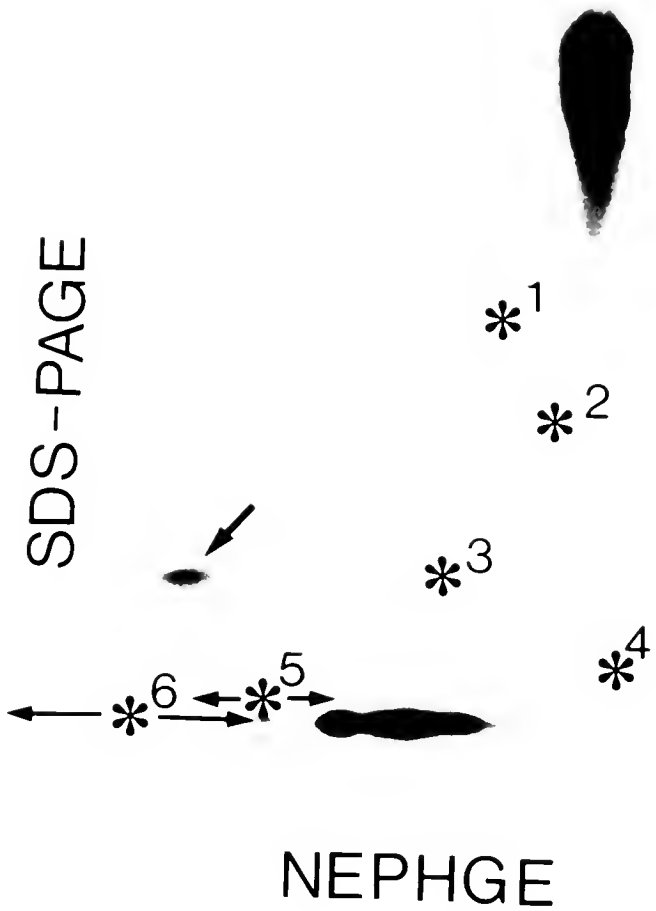


Fig. 2-6 Determination of the isoelectric point of the 28 kDa fragment of MAP-2. An autoradiogram of a two dimensional gel is shown. The first dimension is Nonequilibrium pH gel electrophoresis and the second dimension is sodium dodecyl sulfate polyacrylamide gel electrophoresis with a 7-17% (w/v) gradient of acrylamide. Protein molecular weight/isoelectric point reference standards were: 1) phosphorylase b; 2) bovine serum albumin; 3) carbonic anhydrase; 4) soybean trypsin inhibitor; 5) ribonuclease and 6) lysozyme.

isoelectric point value of 10 for the 28 kDa component, all of these MAP interactions must be strongly influenced by electrostatic charge. Vallee (1982) has clearly demonstrated that 0.35 M sodium chloride can remove MAPs from taxol-stabilized microtubules, and this observation is also in harmony with the notion of ionic interactions between tubules and MAPs. Furthermore, it has been shown that increased phosphorylation of MAP-2 reduces its ability to promote tubulin polymerization (Murthy and Flavin, 1983).

My use of trace labeling of heat-stable MAPs with [^{32}P]ATP and protein kinase reinforces the general utility of this method as first applied by Heimann et al., (1985). The radiolabeled low-molecular-weight thrombin fragment of MAP-2 is more stable than that obtained with trypsin and chymotrypsin, and this stability may also facilitate studies of the stoichiometry and dissociation constants for fragment binding to microtubules, neurofilaments, or actin. Other more approximate methods using intact MAPs and either densitometry or pelleting of assembled cytoskeletal elements (Miyata et al., 1986; Heimann et al., 1985) still require refinement and/or verification.

Finally, the thrombin results underscore the facility and generality of serine protease of MAP-2 into low- and high-molecular-weight fragments. This behavior is reminiscent of proteolytic action on myosin. Furthermore, intact MAP-2 has an isoelectric point of 5.4 (Berkowitz et al., 1977), whereas I found that the tubule/filament-binding domain has a value of 10. This suggests that there must be

significant acidic and basic charge localization in the high- and low-molecular-weight fragments, respectively, of MAP-2. Nonetheless, the biological significance of this protein design feature awaits further understanding of the role of MAP-2 in the cytomatrix.

CHAPTER 3
THE MICROTUBULE-BINDING FRAGMENT OF MAP-2: LOCATION OF THE PROTEASE-ACCESSIBLE SITE

Introduction

The thrombin protease digestion of MAP-2 shown in Fig. 2-1 revealed a very stable 28 kDa fragment. This fragment possessed the sequences responsible for cosedimentation of MAP-2 with microtubules as well as neurofilaments. Since this fragment contained the "active site" of MAP-2, I wanted to learn more about its biochemical properties. It was already known the fragment had a very basic isoelectric point and it was suspected that the basic residues were involved in the binding to the anionic termini of both alpha and beta tubulin. A second interesting point concerning the structure of the fragment was the specificity and stability of the protease digestion products. The autoradiogram of Fig. 2-1 showed no heterogeneity in the 28 kDa microtubule-binding fragment and limited heterogeneity in the very protease-sensitive 240 kDa domain. However, limited heterogeneity in the larger fragment could be observed by the low resolving power of the 15% (w/v) polyacrylamide gel for large molecular weight proteins. Nevertheless, the specificity of the digestion in producing the 28 kDa fragment was impressive. This specificity implied some higher order structure in the MAP-2 molecule yet a previous

report on the circular dichroism spectrum of MAP-2 revealed little alpha-helical or beta-pleated sheet content. If there was no higher order structure, I would expect to see a far more complex pattern of digestion. In order to resove this paradox a large scale purification of the microtubule-binding fragment of MAP-2 was undertaken.

In addition, the structure of MAP-2 was probed by protease digests with and without the presence of microtubules. The low-molecular-weight digestion products were isolated and their amino terminal composition checked by Edman sequencing.

Materials and Methods

Materials

[^{32}P]ATP (7000 Ci/mmol) was purchased from ICN along with ultrapure grades of ammonium sulfate, sodium dodecyl sulfate (SDS), acrylamide, and bis-acrylamide. Immobilon was obtained from Millipore Corporation and coomassie brilliant blue R-250 was from Serva. DEAE-Sephadex A-50 was purchased from Pharmacia; and bovine thrombin, trifluoroacetic acid, Mes buffer, and phenylmethane-sulfonylfluoride (PMSF) were from Sigma.

Preparation of proteins

Bovine brain microtubule protein was prepared by the procedure of Shelanski et al. (1973). MAP-2 was purified by the method of Herzog and Weber (1978) and radiolabeled as previously described (Flynn et al. 1987).

Preparation of heat-stable microtubule-binding fragments

Heat-stable microtubule-binding fragments were prepared according to Vallee (1986) with the following modifications. Thrombin was used instead of chymotrypsin at 8 U/ml and 37°C for 30 minutes to digest thrice cycled bovine microtubule-protein at a concentration of 5 mg/ml. PMSF was added to 1 mM at the end of the digestion to stop proteolysis. The assembled tubules were sedimented at 100,000 x g for 75 minutes at 30°C and the pellet was resuspended in 0.75 M NaCl and 1 mM dithiothreitol. After homogenization and incubation on ice for 30 minutes the protein was heated in a boiling water bath for nine minutes followed by cooling on ice for 20 minutes. The resulting slurry was centrifuged for 30 minutes at 15,000 x g at 4°C. The supernatant fraction contained heat-stable microtubule-binding fragments from tau and MAP-2. These heat-stable binding fragments were concentrated by ammonium sulfate precipitation and then dialyzed against microtubule assembly buffer (100 mM Mes, pH 6.8, 1 mM EGTA, and 1 mM magnesium sulfate) at 4°C with 1 mM PMSF, and passed over a 1 ml DEAE-Sephadex A-50 column equilibrated in the same buffer. The breakthrough fractions were pooled and precipitated with 60% (w/v) ammonium sulfate. After sedimentation, the precipitate was resuspended in assembly buffer and used for HPLC analysis or Immobilon blotting.

Preparation of the 28 kDa fragment of MAP-2 without microtubules present during digestion

Purified MAP-2 corresponding to 0.6 mg at a concentration of 2.0 mg/ml was digested with 5 U/ml thrombin for 30 minutes at 37°C. Digestion was quenched with 2 mM PMSF and cooling on ice for 10 minutes. The digestion products were passed over a 1 ml DEAE-Sephadex A-50 column and handled as described in the preceding section.

HPLC purification of the microtubule-binding fragment of MAP-2 and its amino acid analysis

High performance liquid chromatography was carried out on a Hewlett Packard Model 1090a chromatograph, equipped with a diode array detector. The ammonium sulfate concentrated, microtubule-binding fragments were clarified by centrifugation at 3000 x g for 5 minutes prior to loading on a Waters Associates C-18 column, equilibrated in 0.1% (v/v) trifluoroacetic acid. The protein was eluted with a linear gradient from 0-50% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min and 1 ml fractions were collected. The elution profile was monitored at a wavelength of 220 nm because the fragments are very low in aromatic amino acid content. Initially, polyacrylamide gel electrophoresis was used to check the composition of material in each peak. Fractions containing the MAP-2 microtubule-binding domain were pooled, dialyzed against 100 mM ammonium bicarbonate, lyophilized, and then hydrolyzed in 6 N HCl for 24 hours at 110°C. Samples were analyzed with a Beckman Model 6300 Amino Acid Analyzer.

SDS electrophoresis and blotting

Immobilon was handled according to the manufacturer's instructions prior to electroblotting. The polyvinylidenedifluoride membrane was wetted in 100% (v/v) methanol for 5 minutes followed by soaking in distilled water for another 5 minutes and then it was allowed to dry. Ultrapure grades of SDS, acrylamide, and bis-acrylamide were used to avoid blocking the N-terminus. The electrophoretic samples for sequencing were heated to 80°C for 5 minutes after adding Laemmli sample buffer which contained ultrapure SDS but no bromophenol blue dye. As an indicator of when the electrophoresis was finished molecular weight markers were run in adjacent lanes with dye present. To scavenge any radicals that could possibly react with the samples, 0.1% (v/v) thioglycolate was added to the top chamber buffer. A 12% (w/v) acrylamide SDS-gel containing heat-stable microtubule-binding fragments was electrophoretically transferred to the membrane in 10 mM CAPs, pH 10.0, 10% (v/v) methanol for 6 hours at 70 volts. The membrane was stained with Coomassie Brilliant Blue R-250, destained in 50% (v/v) methanol-10% (v/v) acetic acid. The blot was air dried and stored at -20°C in the dark until the sequencer was available. The band of interest was excised with a razor blade and sequenced in a gas-phase protein sequencer (Applied Biosystems 470A protein sequencer) with on-line phenylthiohydantoin analyzer at the Protein Chemistry core facility.

Results

Site of thrombin cleavage

In order to gain more information about this site of facile thrombin cleavage, a high-yield isolation method was developed for amino acid analysis and sequencing experiments. I again employed thrombin, but digested assembled three-cycle microtubule-protein (*i.e.*, tubulin and MAPs), followed by heat-treatment of the resulting pellet to remove all tubulin and heat-labile MAPs. Then, DEAE-Sephadex ion-exchange chromatography was used to separate the heat-stable microtubule-binding fragments from any high molecular weight digestion products. This ion-exchange chromatography step was a key part of the purification and took advantage of the basicity of the microtubule-binding fragments as they did not interact with the resin while any incompletely digested MAPs and any contaminating projection domain did interact with the resin very strongly. This is shown in Fig. 3-1. The breakthrough fractions from the DEAE-Sephadex chromatography contained the microtubule-binding fragments of the heat-stable proteins MAP-2 and tau (see lane 5) resulting in greater heterogeneity than that seen in Fig. 2-1 where purified MAP-2 was digested. I also found that a tau monoclonal antibody recognized the upper bands in lane 5, identifying them as putative digestion products of tau.

At this point in the purification scheme, I observed four closely spaced bands on a gel ranging from 28 to 36

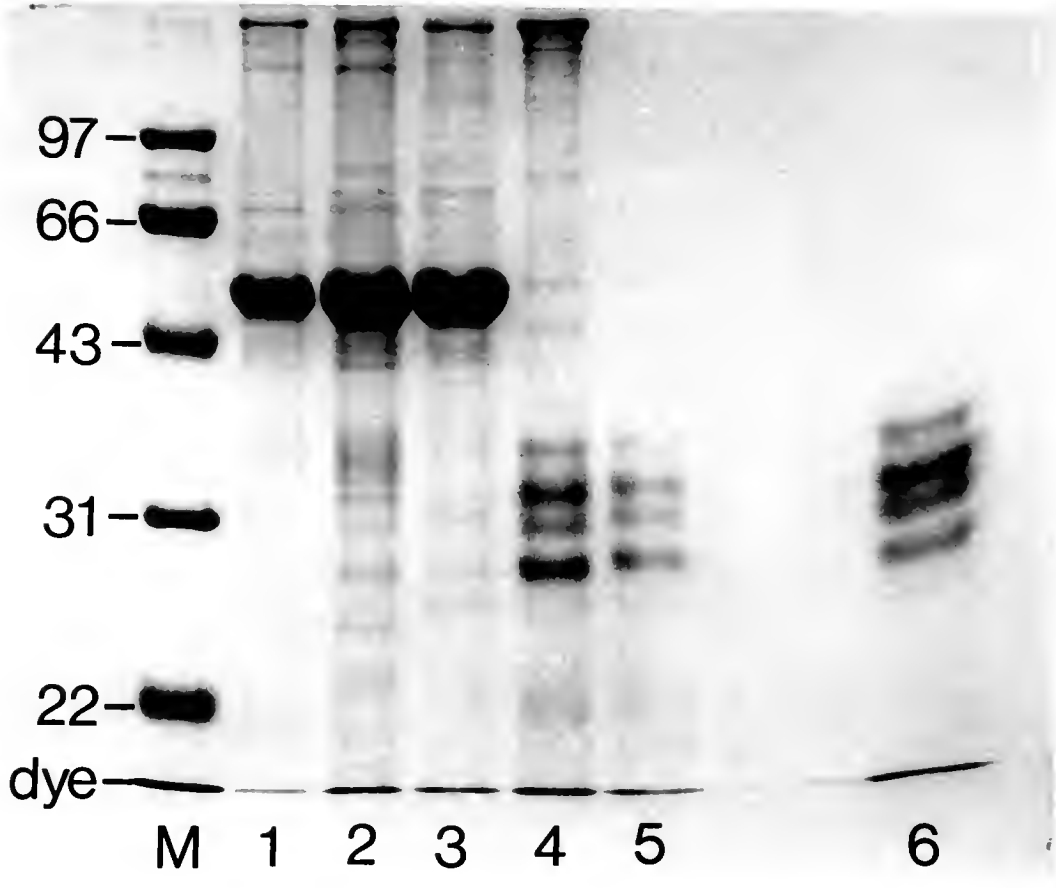


Fig. 3-1 Purification of heat-stable microtubule-binding fragment of MAP-2 and tau. Microtubule-protein was digested with thrombin as described under "Methods". The coomassie blue stain of a 12% (w/v) polyacrylamide gel shows the purification during its various stages. Lane 1 is before cleavage; 2, after cleavage; 3, supernatant after centrifugation; 4, heat-stable protein; 5, after DEAE-Sephadex chromatography; 6, a larger amount of sample 5. M corresponds to molecular weight markers.

kDa. Many different ion-exchange resins were tried to separate these bands such as strong and weak cation exchangers, and hydroxyapatite chromatography, but all were unsuccessful. A two-dimensional gel using the NEPHGE technique in the first dimension was run and all four fragments were found to be very basic but each fragment was slightly less basic with increasing molecular weight. This indicated that the use of ion-exchange resins to separate the fragments was probably futile and another means of separation was necessary. The microtubule-binding fragments in lane 5 of Fig. 3-1 can be readily separated by reverse-phase HPLC as shown in Fig. 3-2 where SDS gel electrophoresis revealed that peak C corresponded to the 28 kDa component. This lane also revealed that there is only minor contamination by a faster migrating component. Fraction C was subjected to acid-catalyzed hydrolysis and amino acid analysis, and these results are listed in Table 3-1. I had previously reported this fragment had an unusually high isoelectric point in comparison with intact MAP-2 (Flynn et al. 1987), and the amino acid analysis confirmed this observation. The fragment is comprised of nearly 14 mole percent in lysyl and arginyl residues. Curiously it contains a higher than usual proline content. The analysis also confirmed the fact that this fragment was low in aromatic amino acids as it contained only two tyrosine residues and three phenylalanine residues. This low content of aromatic residues makes it very difficult to monitor its purification at 280 nm as is usually done for

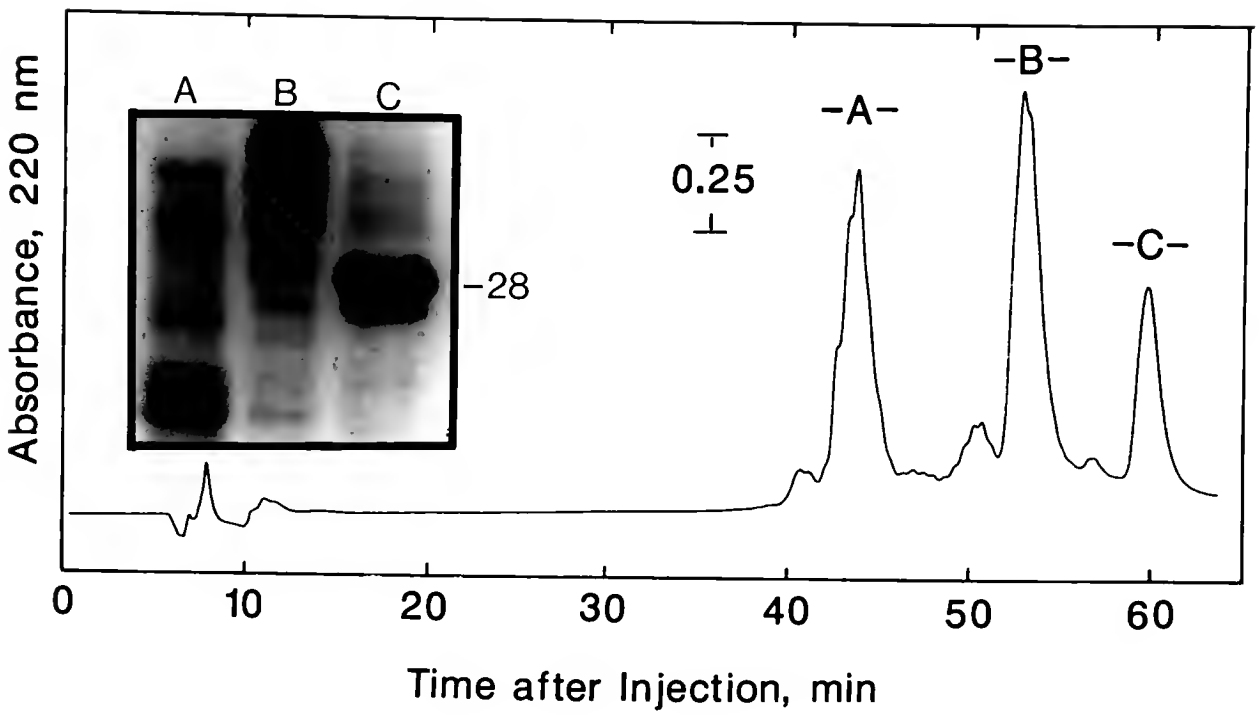


Fig. 3-2 HPLC purification of the microtubule-binding fragment of MAP-2 digested with microtubules present. The MAP-fragments seen in lane 5 of Fig. 3-1 were separated by reverse phase HPLC and the fractions resolved by a 12% (w/v) polyacrylamide gel (see inset) which is coomassie stained.

TABLE 3-1

Amino acid composition of the 28 kDa MAP-2 fragment

Amino Acid	24 h Hydrolysate (mol%)	Estimated Residues/mol
Asx	8.5	21
Thr	5.2	14
Ser	9.7	24
Glx	9.2	22
Pro	6.9	18
Gly	9.0	24
Ala	7.5	18
Val	5.5	15
Met	0.2	0
Ile	4.7	12
Leu	8.1	20
Tyr	0.8	2
Phe	1.4	3
Lys	9.6	25
His	2.9	7
Arg	4.3	11

proteins. Also the analysis was rich in its glutamate + glutamine content as well as its aspartate + asparagine content even though it has already been established the fragment was very basic. This meant that most of these amino acids were in the amide form rather than the carboxylic acid form.

Amino-terminal sequence of the microtubule-binding fragment

Microsequencing techniques were employed with the 28 kDa fragment electroblotted from SDS-polyacrylamide gels to a derivatized nylon screen (Immobilon). This allowed further reduction in any contamination by other MAP fragments such as that observed in the HPLC preparation. The amino terminal sequence obtained was, Thr-Pro-His-Thr-Pro-Gly-Thr-Pro-Lys-Ser-Ala-Ile-Leu-Val-Pro-Ser-Glu-Lys-Lys, based on the results listed in Table 3-2. In the absence of thrombin treatment, identical sequence experiments with either electroblotted MAP-2, as well as MAP-2 in solution, did not yield any phenylthiohydantoin derivatized amino acids at detectable levels. Likewise, experiments with the immobilized 240 kDa projection-arm fragment yielded no sequence data. Protein samples failing to yield detectable levels of amino acid derivatives were subjected to acid-catalyzed hydrolysis and amino acid analysis to assure that sufficient levels of protein for sequencing had been employed. In all cases adequate levels of protein were present above the detection limits for

TABLE 3-2

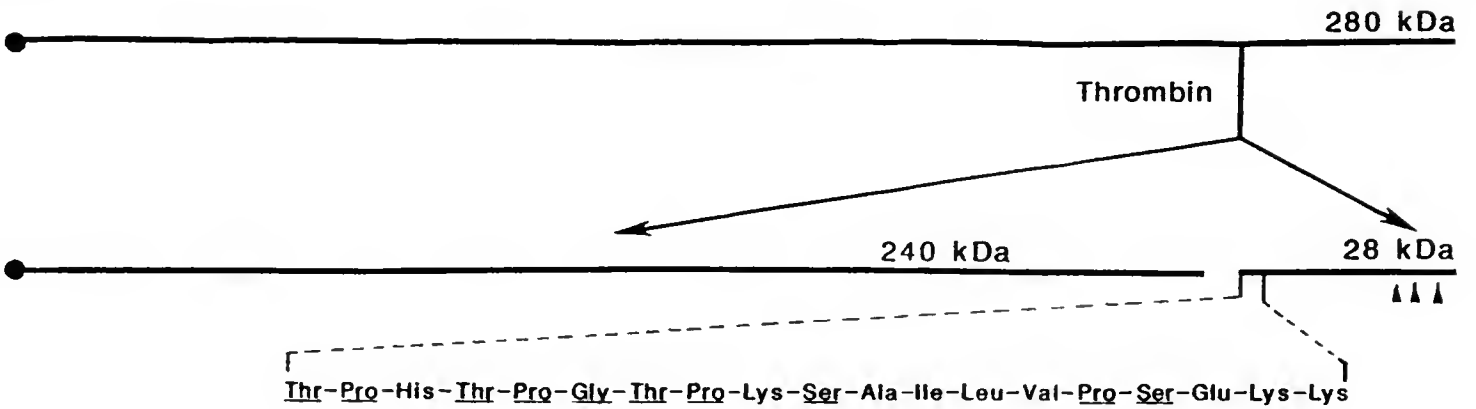
Amino-terminal sequence analysis of the 28 kDa MAP-2 fragment

Cycle No.	Residue	(pmol)[a]	Cycle No.	Residue	(pmol)
1	Thr	74	11	Ala	103
2	Pro	131	12	Ile	74
3	His	15	13	Leu	144
4	Thr	42	14	Val	82
5	Pro	122	15	Pro	102
6	Gly	84	16	Ser	65
7	Thr	54	17	Glu	39
8	Pro	105	18	Lys	105
9	Lys	141	19	Lys	78
10	Ser	85			

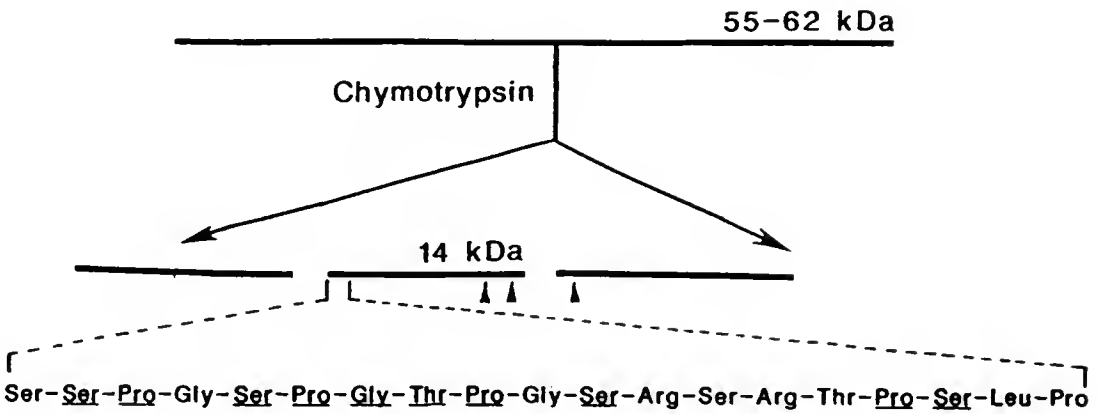
[a] Values reported correspond to the sum of the major, leading, and trailing cycle yields for each amino acid.

Fig. 3-3 Comparison of proteolytic fragmentation patterns and the amino-terminal sequences of the microtubule-binding fragments of MAP-2 and tau protein. The polypeptide chains and cleavage patterns for MAP-2 and tau are represented as heavy lines. The closed circles represent the blocked MAP-2 N-terminus, and the underlined amino acid residues represent identical and/or conserved amino acid residues common to both MAP-2 and tau proteins. The tau protein scheme is based on the data of Aizawa et al. (1988) and Lewis et al. (1988). Arrowheads denote octadecapeptide imperfect repeats.

MAP-2:



Tau Proteins:



sequencing. These findings suggested that the amino-terminus of MAP-2 is blocked and that the 240 kDa fragment is derived from the amino-terminus whereas the 28 kDa fragment resided at the carboxyl end. These observations are in accord with the findings by Kosik et al. (1988) who reported that the N-terminus of MAP-2 appears to be blocked; moreover, Lewis et al. (1988) reported the entire derived amino acid sequence using murine MAP-2 cDNA clones. The primary sequence data with bovine brain MAP-2 correspond to the murine sequence spanning residues 1626 to 1644 with only three exceptions. As shown in Fig. 3-3, there is a similar protease-accessible sequence in the microtubule-binding fragment of bovine tau protein. In that case, however, fragments were generated by chymotryptic cleavage (Aizawa et al., 1988). Both of these cleavage-site sequences reside approximately thirty-to-forty residues toward the N-terminal side of the first of three nonidentical octadecapeptide repeats (indicated schematically by the arrowheads) found in both MAP-2 and tau (Lewis et al., 1988; Lee et al., 1988).

Amino-terminal sequence of the microtubule-binding fragment after digestion in the absence of microtubules

My initial experiments to determine if the microtubule-binding fragment of MAP-2 also bound to neurofilaments, digested purified MAP-2 alone in solution. In the preceding section the amino terminal sequence of the binding fragment was determined but the fragment was generated by digestion of microtubule-protein at 37°C in the presence of 1 mM guanosine triphosphate. In this regard, MAP-2 was digested in the presence of microtubules, a condition that might have

influenced the cleavage point of thrombin were the preferred site of digestion hindered by interaction with the microtubule lattice. To determine whether the cleavage point is the same for the microtubule-binding fragment under the two different conditions, the amino terminal sequence was checked once again but now in the absence of microtubules during the digestion. Purified MAP-2 was cleaved alone in solution, and passed over a DEAE ion-exchange column to remove the projection domain. The breakthrough fractions containing the microtubule-binding domain were pooled, electrophoresed through a polyacrylamide gel, transferred to a polyvinylidenedifluoride membrane, and sequenced by automated Edman chemistry. Ten cycles were performed yielding ten residues that were exactly the same as the first ten residues in Table 3-2 and Fig. 3-3. This indicated that thrombin cleaved MAP-2 at the same argininyl residue in the presence and absence of microtubules.

Additionally, the breakthrough fractions from the DEAE-Sephadex column were subjected to reverse phase HPLC similar to the fragments generated in the presence of microtubules. When loaded on a C-18 column and eluted with the same gradient as mentioned in the preceding section, one major peak corresponding to the 28 kDa fragment was seen (see Fig. 3-4) and one minor peak corresponding to peak A in Fig. 3-2 was seen. The elution time for the 28 kDa fragment was unchanged from Fig. 3-2 and corresponded to peak C from this figure.

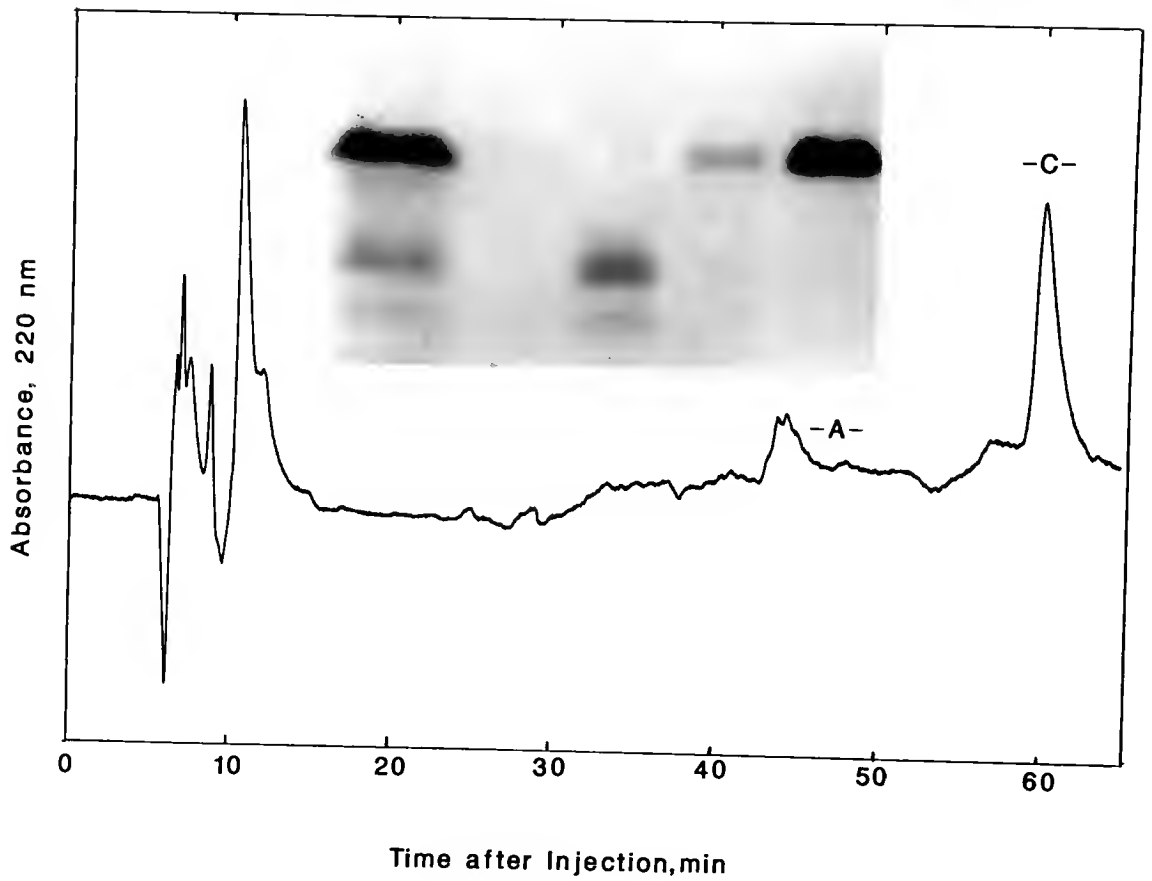


Fig. 3-4 HPLC purification of the microtubule-binding fragment of MAP-2 digested initially without microtubules. The inset shows a 12% (w/v) polyacrylamide gel before and after the HPLC purification: lane 1, digest before purification; lane 2, fraction 22; lane 3, fraction 23; lane 4, fraction 30; lane 5, fraction 31. Fractions were collected every two minutes and were 1 ml in volume.

Discussion

The experiments described in this chapter were designed to gain further insight about the microtubule-binding fragment of MAP-2. There is now general agreement that initial proteolytic cleavage of MAP-2 yields two fragments (Vallee 1980; Flynn et al. 1987). With thrombin, these initial cleavage products corresponding to values of 240 kDa and 28 kDa based on electrophoresis, are quite stable with regard to further degradation. All efforts to sequence Immobilon-linked MAP-2 and the similarly immobilized 240 kDa projection arm fragment consistently failed to yield any detectable levels of PTH-amino acids. Nevertheless, acid hydrolysis and subsequent amino acid analysis of these immobilized proteins demonstrated that sufficient levels were clearly present for detection in the gas-phase sequencer. This observation led me to believe that intact MAP-2 showed no evidence of a free N-terminus, and another group recently reported the same difficulty in attempts to sequence MAP-2 (Kosik et al. 1988). These observations suggest that the MAP-2 amino acid sequence, as derived from nucleotide sequence data (Lewis et al. 1988), does not provide a complete account of the MAP-2 primary structure, and further work will be required to establish the nature of the N-terminal modification blocking Edman degradation.

A striking common structural feature in MAP-2 and tau emerges from the combined findings of Aizawa et al. (1988) and these studies. The former found that chymotryptic

cleavage of the bovine tau proteins yielded a microtubule-binding fragment with the N-terminal sequence shown in Fig. 3-3, and I have now demonstrated that thrombin attacks at a similarly accessible region in bovine MAP-2 (See also Fig. 3-3). It should be noted that both of these cytomatrix proteins have four proline residues in exact registration, and with the exception of the occurrence of a val-pro in the MAP-2 sequence, each of the prolines in both cleavage sites was preceded by a hydroxy-amino acid. Efforts to survey other known sequences in the GenBank database have indicated the uniqueness of these protease-accessible regions in tau and MAP-2; however, Earnshaw et al. (1987) described a centromere-binding protein containing three prolines in exactly corresponding positions with little other structural relatedness to tau and MAP-2. Also it should be noted that the NF-M sequence in chicken contains a proline at every third residue for 102 residues in the repeated sequence $(EXPXSP)_{17}$ (Zopf et al., 1987). The circular dichroism spectral data of Hernandez et al. (1986) indicates that uncleaved MAP-2 contains little, if any, alpha helical or pleated-sheet secondary structure; yet, the preferential action of the endoprotease thrombin at a single site suggests that MAP-2 may display some "hinge-point" behavior akin to the protease-accessible region of myosin. This region may permit the projection arm to swing away from the microtubule surface. Certainly, the observed sedimentation coefficient of 4.5 (Hernandez et al. 1986) also suggests that MAP-2 has extended a flexible structure. The roughly

spherical hemoglobin molecule, itself only one-third the molecular weight of MAP-2, has an almost identical sedimentation coefficient (Sanders et al. 1981). Chymotryptic cleavage between Tyr-128 and Ser-129 in the tau proteins may reflect a corresponding protease-accessible site of structural discontinuity between microtubule-binding and projection domains. These hinge point regions may be very important in their presentation of the microtubule-binding sequences that actually interact with tubulin.

CHAPTER 4
THE MICROTUBULE-BINDING FRAGMENT OF MAP-2: IDENTIFICATION OF AN ASSEMBLY-PROMOTING PEPTIDE AND DISPLACEMENT OF HIGH-MOLECULAR-WEIGHT MAPs

Introduction

Microtubule-associated proteins (MAPs) exhibit one of several properties: the ability to copolymerize with tubulin during microtubule assembly, the capacity to utilize tubulin or another MAP as substrates for enzyme-catalyzed modification, or the use of microtubules as the architectural framework for motility (Olmsted, 1986; Purich and Kristofferson, 1984). The first property is shared by the high-molecular-weight proteins (MAP-1 and MAP-2) as well as the tau proteins, and these proteins remain associated with reassembled microtubules during the course of microtubule-protein purification. Recently, the cDNA-derived amino acid sequences of the murine MAP-2 (Lewis et al., 1988) and the murine tau (Lee et al., 1988) proteins have been defined, and these proteins were both found to contain a related triad of imperfectly repeated octadecapeptide sequences in their tubule-binding regions. Oligopeptide analogues of the repeated sequences in murine tau and a 190 kD bovine adrenal gland MAP can promote microtubule assembly as monitored by light scattering techniques (Ennulat et al., 1989; Aizawa et al., 1989). I wished to investigate whether the triad of imperfect

octadecapeptide repeats of murine MAP-2 and a MAP-2 sequence from the protease-accessible hinge region could promote microtubule polymerization and mimic the action of MAPs.

While several peptides corresponding to sequences in fibrous MAPs can stimulate microtubule assembly, very little is known about whether these synthetic peptides constitute the entire site necessary for the MAP binding to microtubules. At the time little information on MAP-2 sequences reponsible for binding to microtubules or promotion of tubulin polymerization was available. The report of Lewis et al. (1988) showed a 100 residue polypeptide consisting of the first two imperfect repeats plus flanking sequences could cosediment with MAP-stabilized microtubules. No information on the binding of small peptides or promotion of tubulin polymerization was known. The experiments described in this chapter attempt to define an "active site" of MAP-2 by testing for sequences promoting tubulin polymerization. The most likely candidates were the repeated sequences since two of them were in the 100 residue polypeptide of Lewis et al. (1988). These peptides were chemically synthesized along with a hinge-region sequence and tested for stimulation of tubulin polymerization.

If the repeated sequences are indeed the primary sites of interaction, then those promoting tubule assembly in the absence of MAPs may also displace MAPs from microtubules or block their binding to microtubules. Moreover, I was motivated to learn whether a particular peptide and MAP display competitive binding behavior that would indicate the

peptide(s) binding to the same site as MAPs on the microtubule. The effectiveness of all three MAP-2 repeated peptide analogues in terms of MAP displacement from microtubules was also checked. These experiments show that peptides corresponding to the second repeated sequence of MAP-2 can promote microtubule polymerization and displace MAP-1 and MAP-2 from recycled microtubule-protein.

Materials and Methods

Materials

[^{32}P]ATP (7000 Ci/mmol) and [^{3}H]GTP (18 Ci/mmol) were purchased from ICN along with ultrapure grades of ammonium sulfate, sodium dodecyl sulfate (SDS), acrylamide, and bis-acrylamide. Liquid scintillation cocktail 3a70 was obtained from Research Products International. Acetate kinase was a Boehringer Mannheim product, while phosphocellulose resin and GF/F glass fiber filters were from Whatman. Anti-ß tubulin antibody was purchased from Amersham. DEAE-Sephadex A-50 was purchased from Pharmacia; and bovine thrombin, trifluoroacetic acid, Mes, Pipes, and Tris buffers, dithiothreitol, guanosine triphosphate, bovine serum albumin, EGTA, Triton X-100, phenylmethanesulfonylfluoride, catalytic subunit of cAMP-dependent protein kinase, and goat anti-murine IgG Texas red conjugate were from Sigma. t-BOC amino acids and the phenylacetamidomethyl resin were from Applied Biosystems International. Taxol was a gift supplied by Dr. Matthew Suffness at the National Cancer Institute, Bethesda Md.

Preparation of proteins

Isotonic bovine brain microtubule-protein was isolated according to the method of Karr et al. (1979) and stored at -80°C after two cycles of assembly-disassembly. Hypotonic bovine brain microtubule-protein was isolated by the method of Shelanski et al. (1973). Tubulin was prepared according to the method of Kristofferson et al. (1986). [^{32}P]MAP-2 was purified by the procedure of Herzog and Weber (1978) as modified by Flynn et al. (1987) except the purified protein was concentrated by ammonium sulfate precipitation after gel filtration chromatography. Unlabeled MAP-2 was prepared identically except the phosphorylation reaction was omitted prior to the gel filtration column.

Preparation of synthetic peptides

All peptides were made with an Applied Biosystems synthesizer model 430A according to the method of Erickson and Merrifield (1976) with t-BOC protected amino acids and starting with a phenylacetamidomethyl resin. Peptides were cleaved and deprotected using a mixture of hydrogen fluoride, anisole, and dimethyl sulfide in a 9:1:0.3 ratio (v/v) at -10°C for 2.5 hours. After evaporation the resin was washed with cold diethyl ether and extracted into 1 M acetic acid and then freeze dried. Purity was tested by HPLC profile or by gas phase microsequencing. The peptides were stored at -20°C as a lyophilized powder. All synthetic peptides except the m_N peptide were made by Dr. Jan Pohl of the microchemical facility at Emory University. The m_N

peptide was synthesized by the Protein chemistry core facility at the University of Florida.

Microtubule assembly with synthetic peptides

All assembly experiments were done with a GTP-regenerating system (MacNeal et al., 1977) consisting of 2 units/ml of acetate kinase, 10 mM acetyl phosphate, and 0.1 mM [^{3}H]GTP (20 Ci/ml). All assay mixes also contained 1 mM dithiothreitol to maintain reduced sulfhydryls in the peptides. The peptides were weighed out just before use and dissolved in 100 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM magnesium sulfate with 1 mM dithiothreitol. Varying concentrations of each peptide were added to 1.6 mg/ml pure tubulin and 0.4 mg/ml three-cycle microtubule-protein and incubated at 30°C for 30 minutes. The extent of microtubule assembly was monitored by the rapid filtration assay of Maccioni and Seeds (1978) as modified by Wilson et al. (1982). Microtubules were diluted 20X into 100 mM Pipes, pH 6.8, 1 mM EGTA, 1 mM magnesium sulfate, 1% (v/v) glutaraldehyde, 10% (v/v) dimethylsulfoxide, 25% (v/v) glycerol, and 1 mM ATP and kept at 30°C until ready to assay. The diluted mixture was then applied to Whatman GF/F filters on a vacuum filtration device presoaked in the same buffer except no glutaraldehyde was used. Each filter was then washed with 15 ml of the same buffer and the radioactivity was solubilized in 1.5 ml 0.1 N NaOH for 30 minutes followed by addition of scintillation cocktail.

Preparation of microtubule seeds and elongation assay

Seeds were prepared according to Kristofferson et al. (1986). Tubulin at 5 mg/ml was assembled with 1 mM GTP in 80 mM Pipes, pH 6.8, 10 mM magnesium chloride, 1 mM EGTA, in 30% (v/v) glycerol at 37°C for 30 minutes. The microtubules were then sheared with 3 passes through a 22 gauge needle to produce microtubule seeds. The seeds were diluted 100X into 0.5 mg/ml tubulin and varying concentrations of peptides. After 30 minutes at 37°C the samples were handled as described in the preceding section for measuring tritiated guanine nucleotides.

Electron microscopy

Microtubules were diluted 10X into a warmed solution of 1% (v/v) glutaraldehyde in microtubule-assembly buffer and fixed for one minute at room temperature. The fixed samples were placed on a carbon coated Formvar 400 mesh grid and negatively stained with 1% (w/v) uranyl acetate. Grids were observed on a JEOL 100 CX microscope at 50,000X magnification. Samples with and without peptide were processed identically.

Fluorescence microscopy

Microtubules formed in the presence of synthetic peptides were diluted 10X into a warmed solution of 1% (v/v) glutaraldehyde and 0.1% (v/v) Triton X-100 in microtubule-assembly buffer and fixed for 2 minutes at room temperature. The microtubules were diluted to 50,000 times their original concentration and an aliquot of 100 μl was centrifuged on to a glass coverslip at 30 psi in a Beckman airfuge for 20

minutes. The coverslip was then fixed in -20°C methanol for 4 minutes and blocked with 10 mg/ml bovine serum albumin in phosphate buffered saline, pH 7.3, with 0.1% (v/v) Triton X-100 for 10 minutes. The coverslip was then stained with a murine anti-tubulin antibody at a dilution of 1:200 for 30 minutes followed by washing in the same buffer. A goat anti-murine IgG secondary antibody conjugated with Texas red fluorochrome was used at a dilution of 1:80 followed by washing in phosphate buffered saline, pH 7.3, with 0.1% (v/v) Triton X-100. The coverslips were mounted in 20 mM Tris, pH 7.9, with 90% (w/v) glycerol and viewed with a Zeiss epifluorescence microscope at 1000X power.

Isotonic microtubule experiments

Before use the isotonic bovine brain microtubule-protein was carried through a third cycle of assembly/disassembly, and the concentration of protein was determined by the method of Bradford (1976). Synthetic peptides were weighed out just prior to use and dissolved in PEM buffer (100 mM Pipes, pH 6.8, 1mM EGTA, 1 mM $MgSO_4$) containing 1 mM dithiothreitol. Peptides were added at the indicated concentrations to 0.8 mg/ml isotonic microtubule-protein with 0.5 mM GTP and 1 mM dithiothreitol and incubated at 37°C for 20 minutes. The microtubules were subsequently stabilized with 10 μM taxol for 10 minutes at 37°C. The samples, 250μl final volume, were then centrifuged for 8 minutes at 300,000 x g, 37°C in a Beckman TL 100.2 rotor. The pellets were dissolved in 8 M urea and analyzed by gel electrophoresis.

Competition with radiolabeled MAP-2

All radiolabeled MAP-2 experiments were performed with polypropionate airfuge tubes which were coated with 10 mg/ml bovine serum albumin for 5 minutes and rinsed with PEM buffer just prior to use. This treatment reduces nonspecific binding of proteins to the walls of the centrifuge tubes. The radiolabeled MAP-2 was clarified prior to use for 20 minutes at 130,000 x g in a Beckman airfuge to remove any aggregated or denatured protein. Phosphocellulose-purified tubulin was incubated at 5 mg/ml, 37°C with 1 mM GTP for 20 minutes and subsequently stabilized with 50 μM taxol for an additional 10 minutes. The microtubules were then diluted twenty-fold into a solution containing 3 μM radiolabeled MAP-2 with either unlabeled MAP-2 or synthetic peptides for 20 minutes at 37°C. The solution also contained 10 μM taxol and 1 mM GTP to maintain microtubule stability. The samples, final volume of 100μl, were then carefully loaded into coated airfuge tubes with the aid of a microcapillary pipetter onto a 50 μl layer of 20% (w/v) sucrose in PEM buffer warmed to 37°C. The samples were centrifuged for 30 minutes at 130,000 x g and the supernatants removed and discarded. The pellets were washed with 100 μl of 10 mg/ml bovine serum albumin in phosphate buffered saline, pH 7.3, containing 0.1% Triton X-100 and resuspended in 100 μl 8 M urea. Aliquots of 25 μl were taken for liquid scintillation counting.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was done by the method of Bloom et al. (1985) omitting sodium dodecyl sulfate in the separating and stacking gels and adding 2 M urea to the separating gel. Gels were stained with coomassie Brilliant Blue R-250 and scanned with an LKB ultrascan densitometer.

Results

Peptide interactions with tubulin and microtubule-protein

To further analyze sequence(s) responsible for MAP-2 binding to tubulin within the 28 kDa fragment, four peptides were synthesized. The first (m_N = TPHTPGTPK) corresponded to the N-terminus of the 28 kDa fragment from bovine MAP-2 that was determined previously by microsequencing (see Table 3-2). The others corresponded to the three octadecapeptide repeats (m_1 = VKSKIGSTDNIKYQPKGG, m_2 = VTSKCGSLKNIRHRPGGG, m_3 = AQAKVGSLDNAHHVPGGG). Peptide m_N was based on the bovine sequence data while the murine MAP-2 sequence data was used for m_1, m_2, and m_3, because no such data are yet available for the bovine MAP-2. The high state of purity of each peptide was confirmed on the basis of HPLC elution profile analysis or gas-phase microsequencing.

First, I sought to determine whether any of these peptides would influence the assembly of microtubule-protein that contained both tubulin and MAPs. I worked with recycled microtubule-protein to which sufficient pure tubulin was added to lower the content of MAPs to about one-fifth their normal level. This final composition was

approximately 5% MAPs and 95% tubulin by weight. This ratio was chosen to accentuate any stimulatory effects of the peptides on the assembly process, and no microtubule polymerization occurred at the protein concentrations used without peptide addition. To assay the extent of microtubule assembly at different levels of peptides m_N, m_1, m_2, and m_3, [^{3}H]guanine nucleotide uptake was measured with the glass fiber filter assay of Maccioni and Seeds (1978) as modified by Wilson et al. (1982). Only peptide m_2, corresponding to the second repeat in MAP-2, stimulated microtubule-assembly as evidenced by the data shown in Fig. 4-1. The level of m_2 peptide required for polymerization was very high. Concentrations greater than 250 μM were needed to stimulate microtubule polymerization. When peptides m_N, m_1, or m_3 were employed individually, no incorporation of guanine nucleotide was observed above background levels. Moreover, in companion experiments, I found that none of these peptides mixed individually with peptide m_2 altered the stimulation of microtubule assembly by peptide m_2. A range of concentrations from 0-1 mM was tried for peptides m_1 and m_3 with 0.5 mM or 0.75 mM m_2 peptide but none showed any effect on the extent of microtubule polymerization.

I also found that assembly of pure tubulin could be stimulated by m_2 only. Indeed, assembly with tubulin and m_2 exhibits a typical time-course for the polymerization process as shown in Fig. 4-2. An initial lag phase indicative of subunit nucleation was seen followed by a

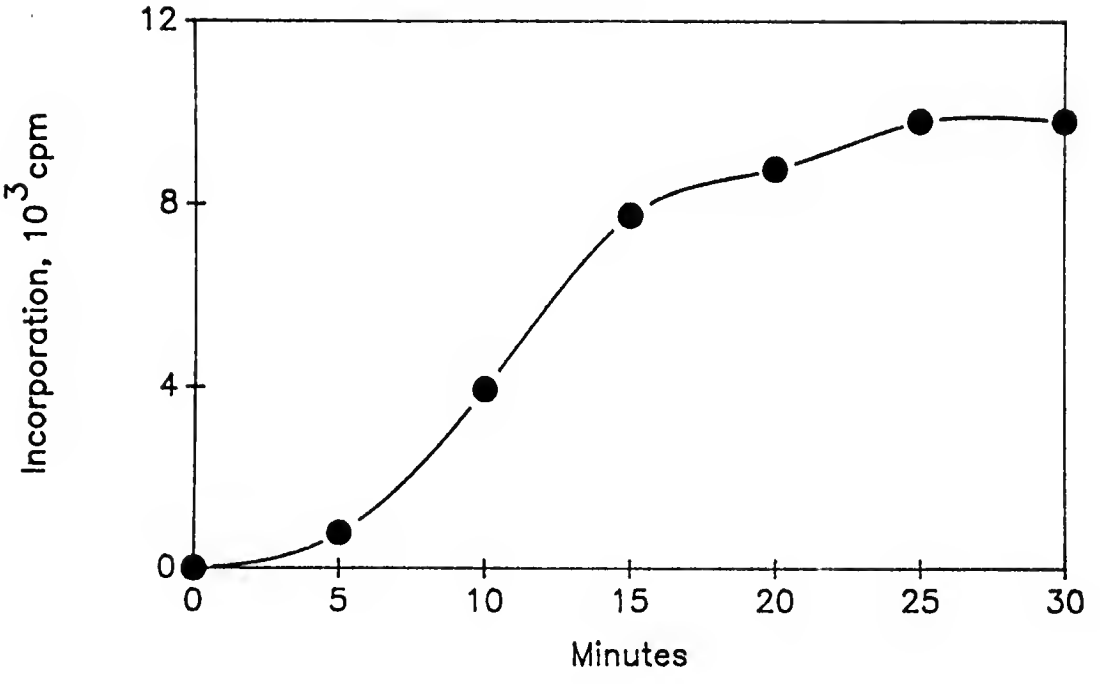


Fig. 4-2 Time course of peptide induced assembly. Phosphocellulose-purified tubulin (1.0 mg/ml) was incubated with m_2 peptide (1.0 mM) for 30 min. at 37°C. At the times indicated, the amount of GTP incorporation was determined as described in "methods".

rapid polymerization phase that plateaued around 30 minutes. Without addition of m_2 peptide, no tritium label is retained on the glass fiber filters. I verified that the observed polymerization resulted in microtubules by using electron microscopy (see Fig. 4-3) and immunofluorescence microscopy (see Fig. 4-4). The electron micrographs revealed a morphology typical of microtubules composed solely of tubulin. When tubulin (1 mg/ml) was incubated with and without peptide m_2 (1 mM), microtubules were observed only in those micrographs of samples to which this peptide had been added. The same was seen for the immunofluorescence micrographs in Fig. 4-4 where panel A had the same concentrations of protein and peptide as the electron micrograph in Fig. 4-3 and panel B was without added peptide. Panel A shows typical *in vitro* microtubules stained with an anti-tubulin antibody. The concentration of tubulin used was 1 mg/ml because it was clearly above the critical concentration for peptide m_2 induced assembly while for tubulin alone it was just at the lower limit for polymerization (see Fig. 4-5). Any molecule that shifts the x-intercept to the left is a microtubule-stabilizer and any molecule that shifts it to the right is a microtubule-destabilizer. Clearly, m_2 is a stabilizer of microtubule polymerization. I also tested the action of several common inhibitors of microtubule assembly to learn whether peptide m_2 induced assembly in a manner akin to normal assembly of brain microtubules. Inclusion of colchicine (0.1 mM),

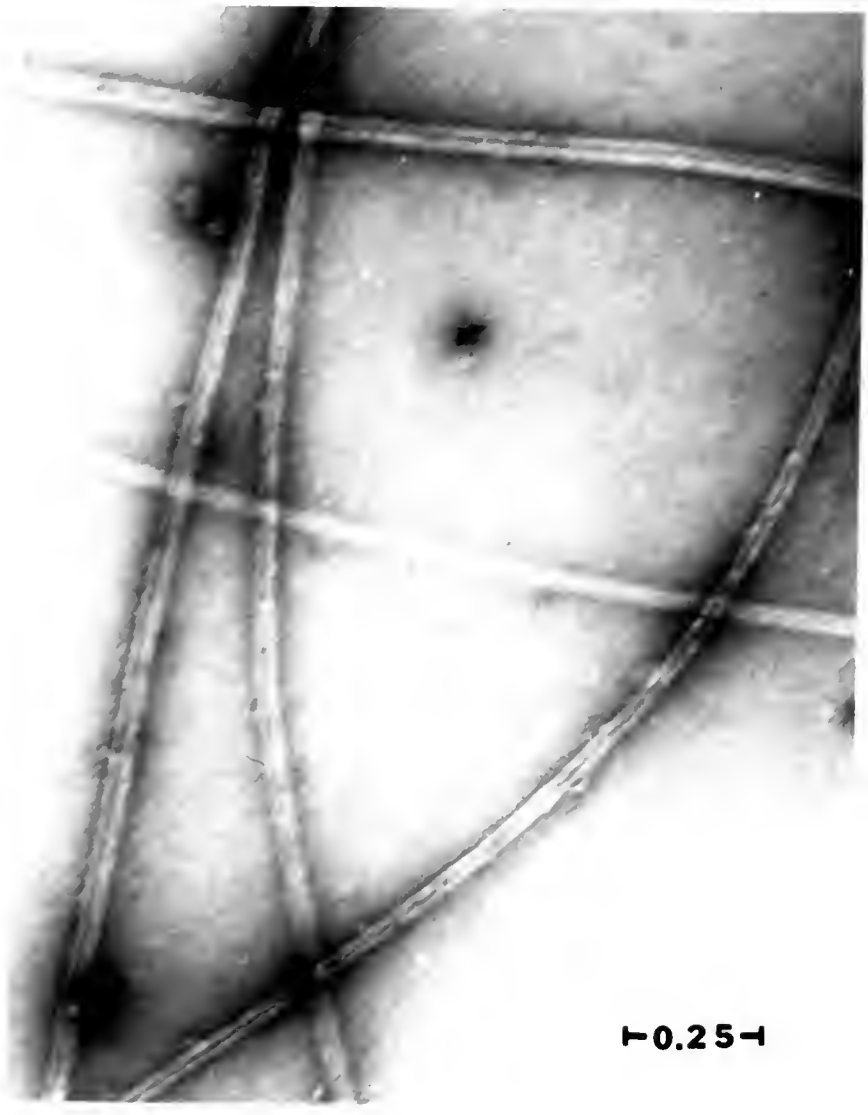


Fig. 4-3 Electron micrograph of peptide induced assembly. Tubulin (1.0 mg/ml) and m_2 peptide (1.0 mM) were incubated for 30 min. at 37°C and then diluted into 1% glutaraldehyde in microtubule assembly buffer warmed to 37°C. After fixation for one minute, the sample was processed for electron microscopy. An identical sample without m_2 peptide was also done but showed no microtubules. Magnification is 50,000X and the bar equals 0.25 μm. Formvar coated grids, uranyl acetate, and photographs were supplied by the Electron Microscopy core facility

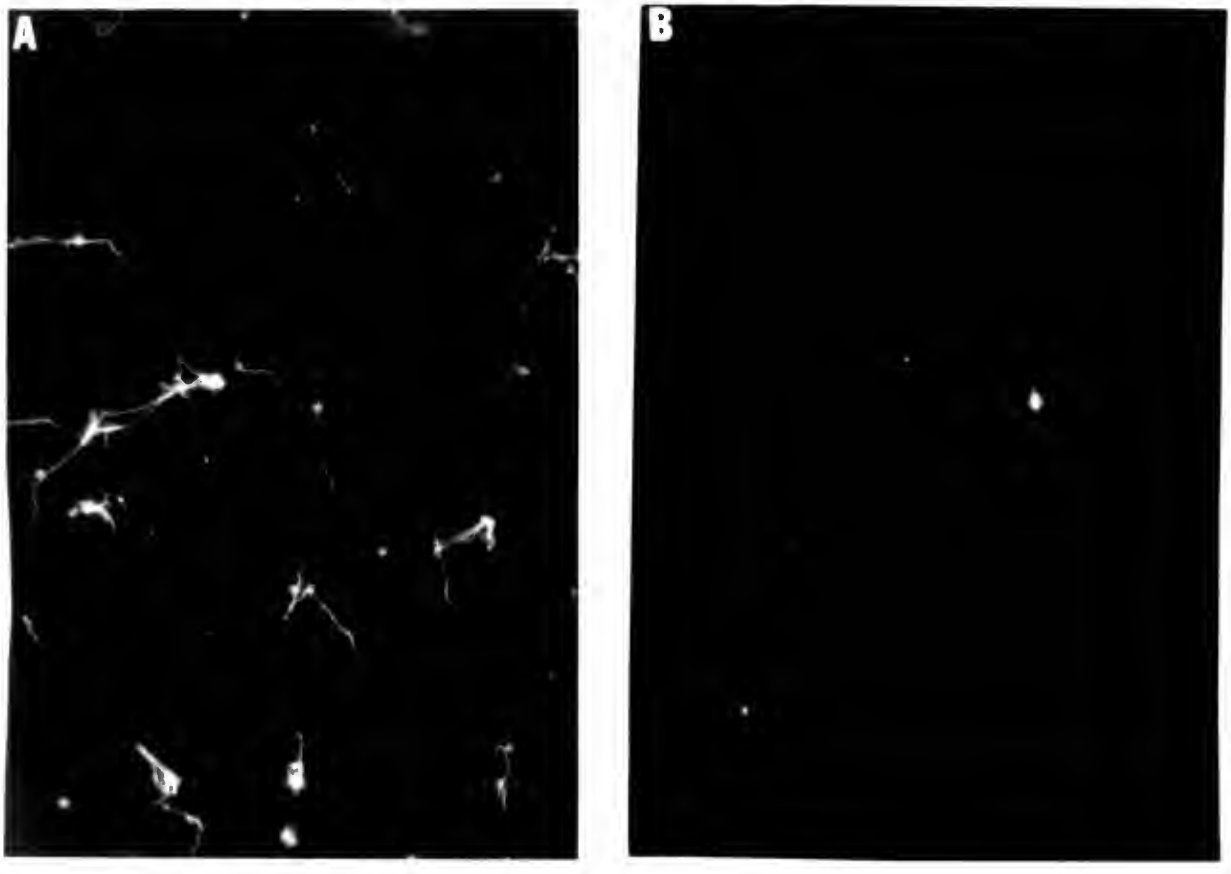


Fig. 4-4 Immunofluorescence of microtubules polymerized with and without m_2 peptide. Panel A shows the same tubulin and peptide concentrations as Fig. 4-3 and panel B shows just tubulin with no peptide addition. Both samples were stained with anti-beta-tubulin followed by an anti-mouse IgG conjugated with Texas red fluorochrome.

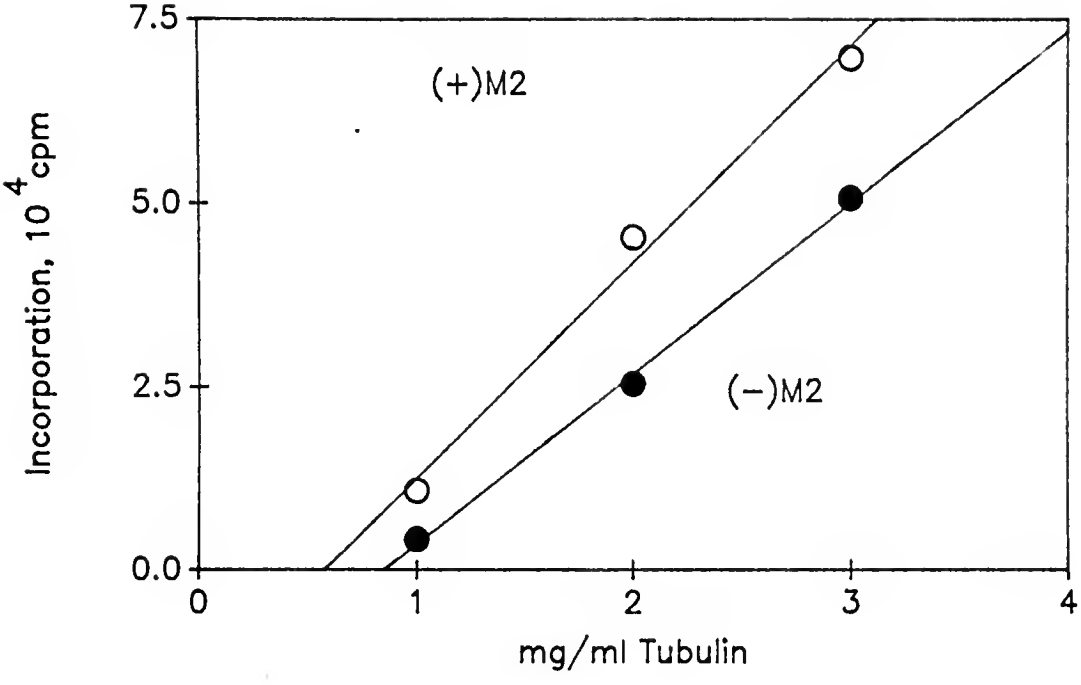


Fig. 4-5 Critical concentration plot of peptide induced tubulin polymerization. Varying concentrations of tubulin plus microtubule seeds were mixed with or without m_2 peptide (1.0 mM) and assayed for GTP incorporation after 30 min. at 37°C.

calcium ion (2 mM), or podophyllotoxin (0.1 mM) resulted in complete inhibition of peptide m_2-induced assembly.

These observations indicate that only the peptide m_2, with a sequence corresponding to the second repeated region of the microtubule-binding fragment MAP-2 could stimulate tubulin assembly. Nonetheless, the possibility remained that the other peptides could still promote elongation, but not nucleation, of microtubule assembly. To investigate this possibility, I added pre-formed microtubule seeds to tubulin (0.5 mg/ml) and [^{3}H]GTP in the presence or absence of the peptides. Without any peptide additions, only a minimal increase in guanine nucleotide incorporation was observed; however, upon addition of peptide m_2, significant assembly was again observed. By contrast, peptides m_1 and m_3 failed to cause any significant increase of labeled guanine nucleotide incorporation into microtubules beyond background levels (see Fig. 4-6). Thus, m_2 is the only peptide that can stimulate nucleation and elongation.

Displacement of MAPs from recycled microtubule-protein by MAP-2 repeated sequence peptides

While only peptide-m_2 promoted microtubule self-assembly, I was interested in determining whether m_1 and m_3 might also bind to assembled tubules and displace MAP-2. Therefore, the ability of the MAP-2 repeated sequence peptides to displace high-molecular-weight MAPs from assembled microtubules was assessed. For this purpose, microtubule-protein isolated by the isotonic extraction method (Karr et al., 1979) was used because such protein as

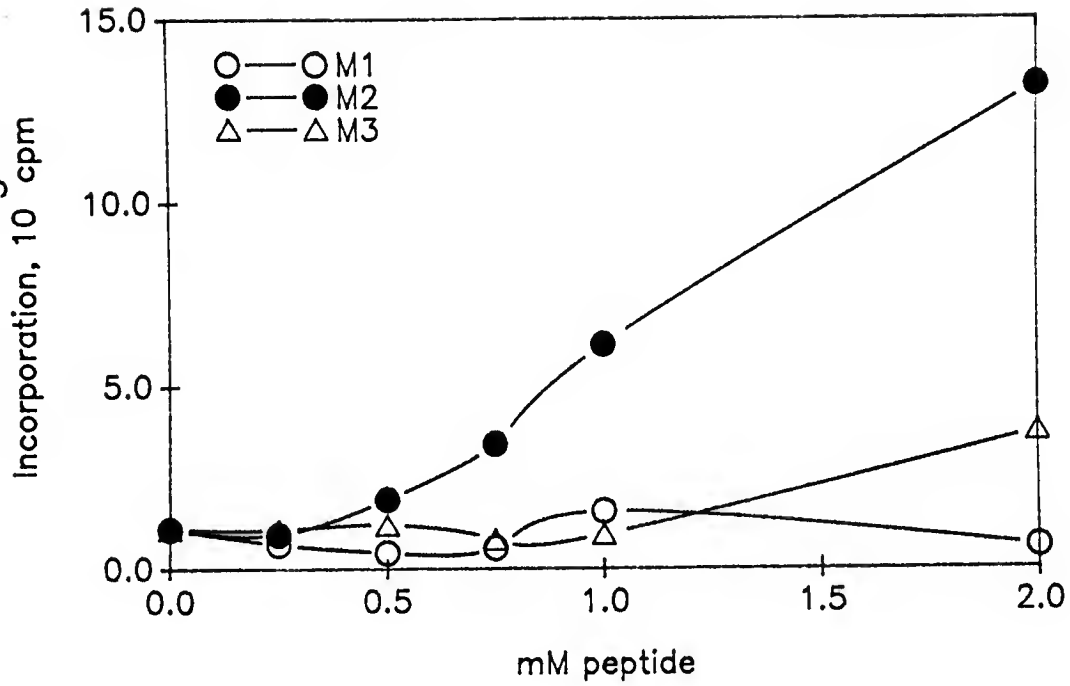


Fig. 4-6 Seeded assembly of tubulin with synthetic peptides. Microtubule seeds were added to a solution containing 0.5 mg/ml tubulin (a level below the critical concentration). Varying amounts of m_1, m_2, and m_3, were added and polymerization initiated by warming to 37°C. The plotted values correspond to radiolabel incorporation over a 30 minute period.

isolated from the gray matter of the brain is rich in both MAP-1 and MAP-2. The isotonic microtubule-protein was stabilized with taxol after 20 minutes of 1 mM GTP at 37°C. This method reduced the amount of abnormal microtubule structures common with taxol-induced polymerization where taxol is added at the start of polymerization. In this experiment, taxol was added after 20 minutes of microtubule assembly. After a 10 minute incubation, the peptides were added and the samples kept at 37°C for an additional 20 minutes. In addition to the three repeats of MAP-2, two other peptides were tested. The first (m_1') was a glycine substitution for a lysine in the m_1 peptide converting the carboxyl terminal sequence to that of all the other repeats. The second (m_2') was the same as m_2 plus the next three residues present in the MAP-2 sequence. These additional residues were RVK which made the peptide more electro-positive as well as possibly adding more structural conformation. As seen in Fig. 4-7, SDS gel electropherograms of the microtubule after assembly and centrifugation, indicate that MAP-2 was only susceptible to displacement by a 21-amino acid peptide m_2' corresponding to the m_2 sequence above plus residues RVK at the C-terminus. Interestingly, MAP-1b was selectively displaced by peptide m_2, and all high-molecular-weight MAPs were removed from microtubules in the presence of peptide m_2'. Densitometry tracings of lanes 2, 5, and 6 indicate the profiles of MAPs with m_2, m_2', and no peptide, respectively (Fig. 4-8). Because the 21-amino acid peptide was more effective in

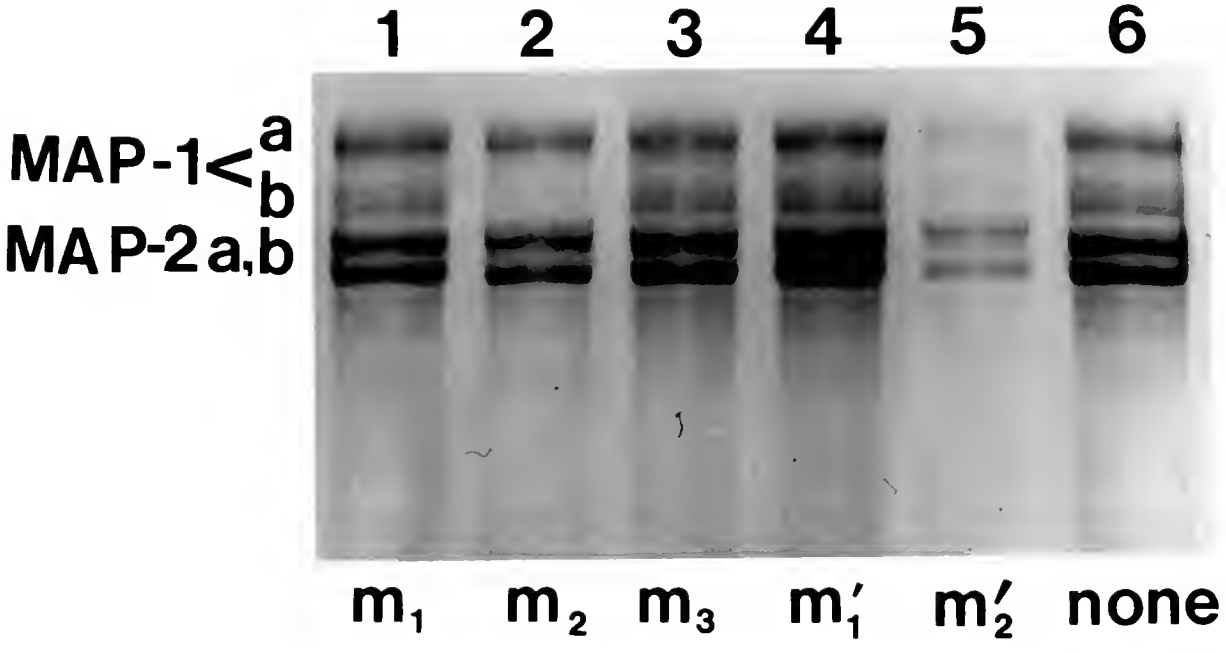


Fig. 4-7 Effects of MAP-2 peptides on MAP binding to microtubules. Coomassie Blue staining of proteins in pelleted microtubule fractions after electrophoresis on a 4% polyacrylamide gel: (Lane 1) m_1; (lane 2) m_2; (lane 3) m_3; (lane 4) m_1'; (lane 5) m_2'; (lane 6) no peptide. All peptides were added to a concentration of 2.0 mM.

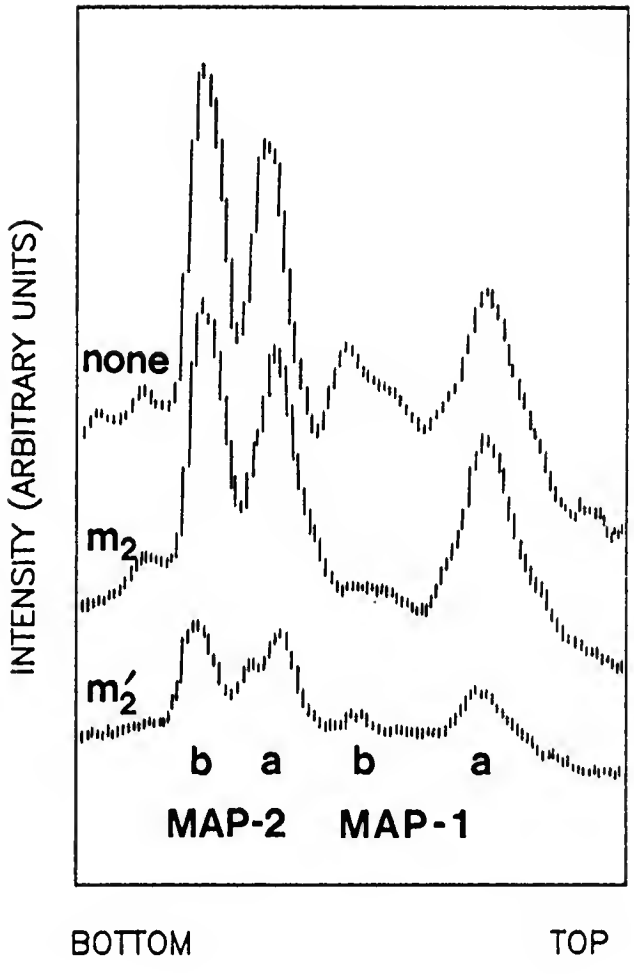


Fig. 4-8 Densitometry of the coomassie blue stained gel. Upper trace represents lane 6, middle trace lane 2, and the lower trace is lane 5 of the gel depicted in Fig. 4-7.

displacing MAPs, it was compared to the m_2-peptide with regard to the promotion of microtubule assembly using tritiated GTP incorporation as a measure of polymerized protein (see Fig. 4-9). I found that tubulin polymerization was considerably more effective in the presence of m_2', especially at lower peptide concentrations. At the greatest concentration of peptide, the level of tritiated GTP incorporation was the same for both m_2 and m_2'. Together, these observations suggest that only peptides corresponding to the second repeated sequence can displace MAPs from assembled microtubules. The data also indicate that both MAP-1 and MAP-2 can be displaced by peptide-m_2', suggesting further that this peptide may bind to common, or closely overlapping, sites on microtubules. Peptides m_1, m_2, and m_3 were otherwise without effect, as was an analogue of m_1 containing a gly in place of lys toward the C-terminus. This m_1 analogue was synthesized because the lysine residue was disrupting a possible beta turn structure. In m_2 and m_3 as well as all three repeats of tau, the carboxyl termini are proline-$(glycine)_3$. This structure was hypothesized to be important for microtubule-assembly since both the first two tau repeats contained this carboxyl terminal tail and could promote tubule polymerization. The first repeat of MAP-2 was very similar to the first repeat of tau except for the glycine difference; however, the m_1 analogue failed to promote tubulin polymerization and also failed to displace high-molecular-weight MAPs.

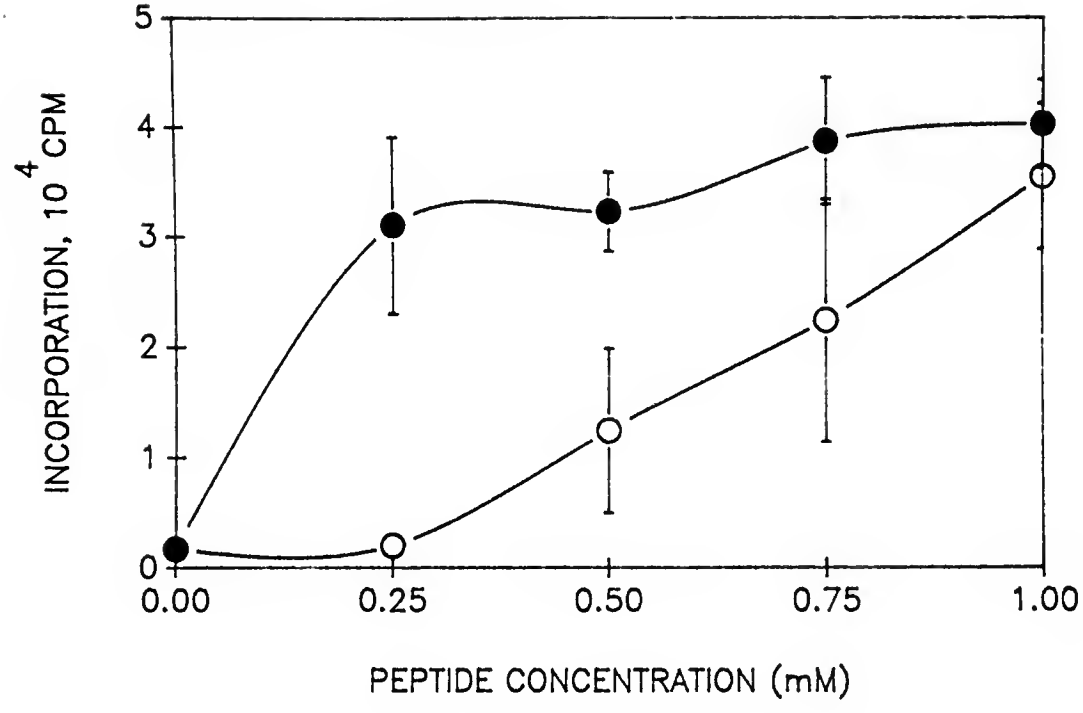


Fig. 4-9 Comparison of the stimulation of tubulin polymerization by peptides m_2 and m_2'. The incorporation of tritiated GTP into microtubule-polymer was measured to examine the assembly promoting activity of m_2 and the extended analogue m_2'. Phosphocellulose-purified tubulin was used at 1 mg/ml. The open circles represent m_2 and the closed circles m_2'.

A gradient of m_2' peptide was used to determine the effective concentration range of displacing the MAPs. The results shown in Fig. 4-10 demonstrate that the extended second repeated sequence peptide m_2' removes MAP-1 and MAP-2 from microtubules in a concentration-dependent manner. Again, densitometry was used to gauge the extent of MAP depletion in the assembled tubule fraction, and the concentration of peptide-m_2' that displaces 50% of MAP-2 was about 1.5-2.0 mM (data not shown). This level of peptide-m_2' is about four times the concentration needed to promote tubulin polymerization in the absence of MAPs or microtubule-stabilizing agents.

Radiolabeled MAP-2 binding to microtubules

While the findings presented in Figs. 4-7 and 4-10 provide clear evidence of MAP displacement, a quantitative displacement/binding assay was developed to more accurately measure the MAP displacement. MAP-2 was incubated with 3',5'-cyclic-AMP-stimulated protein kinase and [γ-^{32}P]ATP under conditions that have been found to result in the incorporation of about 1-1.5 phosphoryl groups per MAP-2 molecule (Flynn et al., 1987). First I determined the concentrations of tubulin and MAP-2 necessary for saturation of binding to microtubules. The level of tubulin used was 0.25 mg/ml which was taxol-stabilized like the isotonic microtubule-protein described in the previous section. At this low level of tubulin, 1-5 μM of ^{32}P-MAP-2 was seen to saturate the available binding sites on the microtubules. This is depicted in Fig. 4-11.

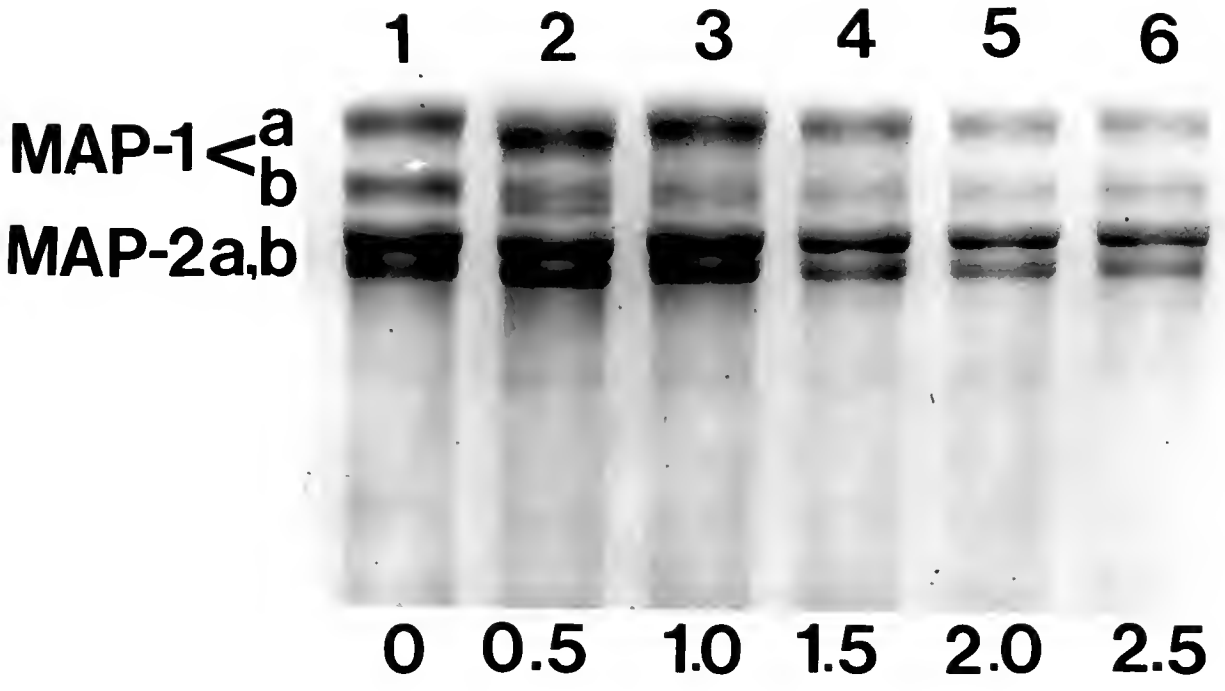


Fig. 4-10 Effects of increasing the m_2' concentration on high-molecular-weight MAP binding to microtubules. Peptide m_2' was added to isotonic microtubule-protein to the final mM concentration indicated at the bottom of each lane. After centrifugation, the pellet fractions were analyzed by gel electrophoresis. The coomassie Blue staining of a 4% polyacrylamide gel is shown.

Because extensive MAP-2 phosphorylation can alter the affinity of MAP-2 to microtubules (Murthy and Flavin, 1983; Hoshi et al., 1988), I wanted to compare the binding behavior of the trace phosphorylated MAP-2 with unmodified MAP-2 isolated by the standard recycling preparation protocol (Shelanski et al., 1973; Herzog and Weber, 1978). Accordingly, taxol-stabilized microtubules were incubated with the phosphorylated MAP-2 in the presence of several concentrations of unphosphorylated MAP-2 (Fig. 4-12). The data points show that the amount of radiolabeled MAP-2 bound to microtubules decreases at increasing concentrations of the unlabeled MAP-2; the solid line is the theoretical curve calculated on the basis of isotopic dilution, using the ratio $R = [MAP\text{-}2^*]/([MAP\text{-}2] + [MAP\text{-}2^*])$, where labeled and unlabeled protein are MAP-2* and MAP-2, respectively. The data normalized with respect to R (Fig. 4-12 inset) indicate that the relative affinities of both MAP-2 species are the same within experimental error.

Displacement of labeled MAP-2 by a second repeated sequence peptide

To gain a more quantitative view of MAP-2 displacement, the amount of microtubule-bound [^{32}P]MAP-2 as a function of the concentration of peptide-m_2' was examined. As shown in Fig. 4-13, MAP-2 is displaced by this peptide, but the desorption process is not described by a typical hyperbolic dissociation curve. The basis of the slight stimulation of MAP-2 binding at 0.5 mM peptide-m_2' is unclear, but careful inspection of the MAP-2 band in lane 2 of Fig. 4-10 revealed

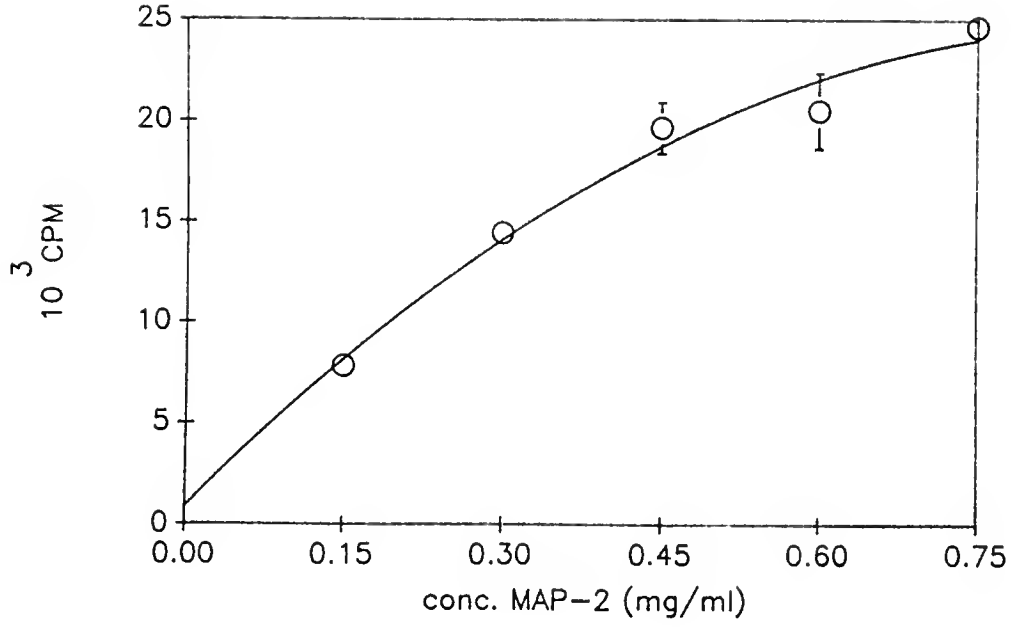


Fig. 4-11 ^{32}P-MAP-2 binding to taxol-stabilized microtubules. Taxol-stabilized microtubules were diluted to 0.25 mg/ml in increasing concentrations of radiolabeled MAP-2. After incubation at 37°C for 20 minutes and centrifugation, the level of MAP-2 binding was determined by liquid scintillation counting.

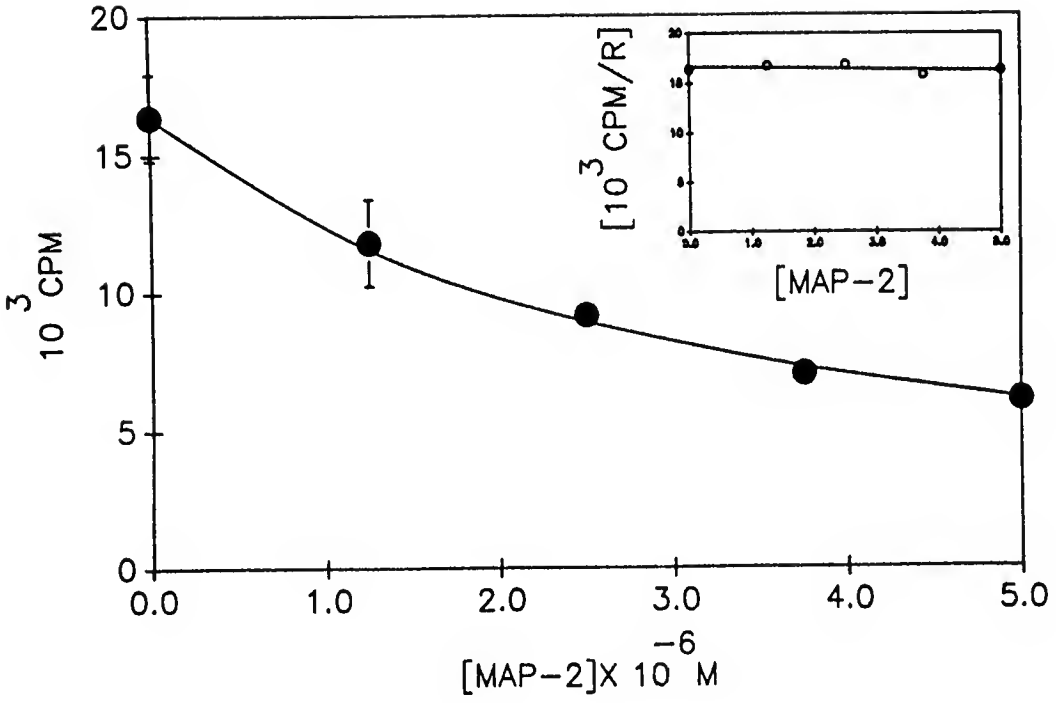


Fig. 4-12 Displacement of trace phosphorylated MAP-2 from taxol-stabilized microtubules by unlabeled MAP-2. See "Competition with Radiolabeled MAP-2" under the "Methods" section. The molarity of MAP-2 was calculated using a molecular weight of 200,000 daltons.

a similar behavior. Once again the effective concentration range for displacement was in the low millimolar range comparable to the SDS gels shown in Fig. 4-10. Thus, the same displacement behavior is observed whether or not MAP-2 is trace phosphorylated by the 3',5'-cyclic AMP-stimulated protein kinase.

Competitive binding of peptide-m_2' and MAP-2

I was particularly interested in the mode of inhibition of MAP-2 binding to microtubules by the extended second repeated sequence peptide-m_2'. Therefore a series of radiolabeled MAP-2 binding measurements were conducted over the concentration range of MAP-2 shown in Fig. 4-14. These experiments were carried out in the absence or presence of 1.5 mM peptide-m_2'. The results indicated that peptide-m_2' did indeed act as a competitive inhibitor of MAP-2 binding as binding of MAP-2 to microtubules was reduced in the presence of the peptide as opposed to without the peptide. When the data was transformed into a double reciprocal plot, the lines intersected at the y-axis (see Fig. 4-15) suggesting the peptide was a competitive inhibitor of MAP-2 binding to microtubules. The data presented here also indicated that the MAP-2 interaction with microtubules was defined by a single class of binding sites. The maximal extent of MAP-2 binding was found to correspond to one molecule of MAP-2 per four molecules of polymerized tubulin dimers. The dissociation constant for MAP-2 binding to microtubules was 3.4 μM in the absence of peptide-m_2' and 14 μM in the presence of 1.5 mM of this peptide. From the

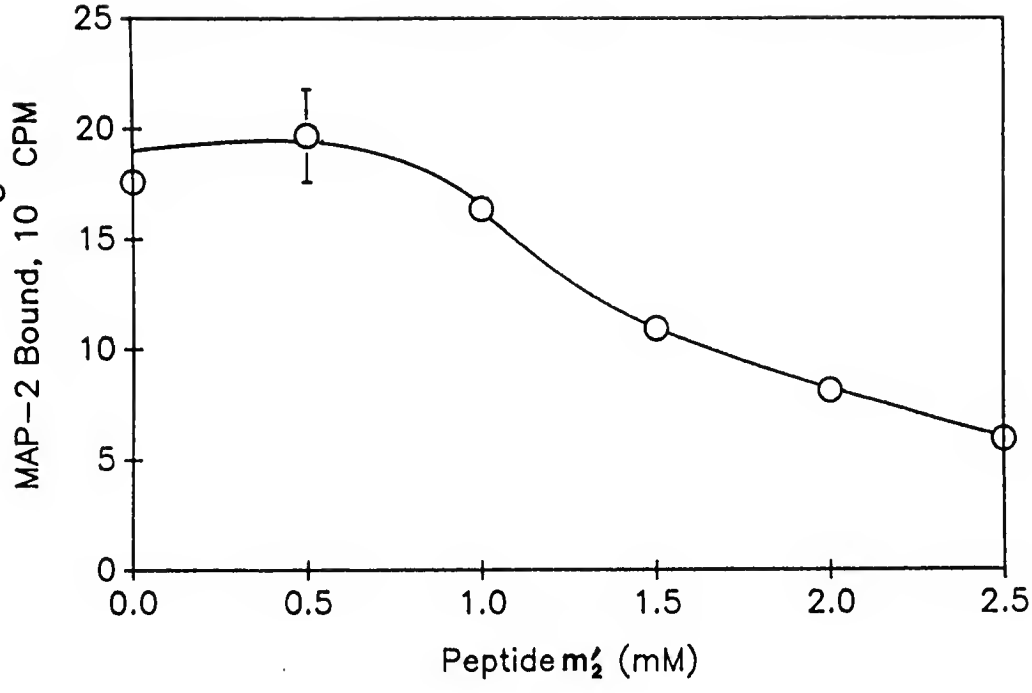


Fig. 4-13 Displacement of trace phosphorylated MAP-2 from taxol-stabilized microtubules by peptide m_2'. See "Competition with Radiolabeled MAP-2" in the "Methods" section.

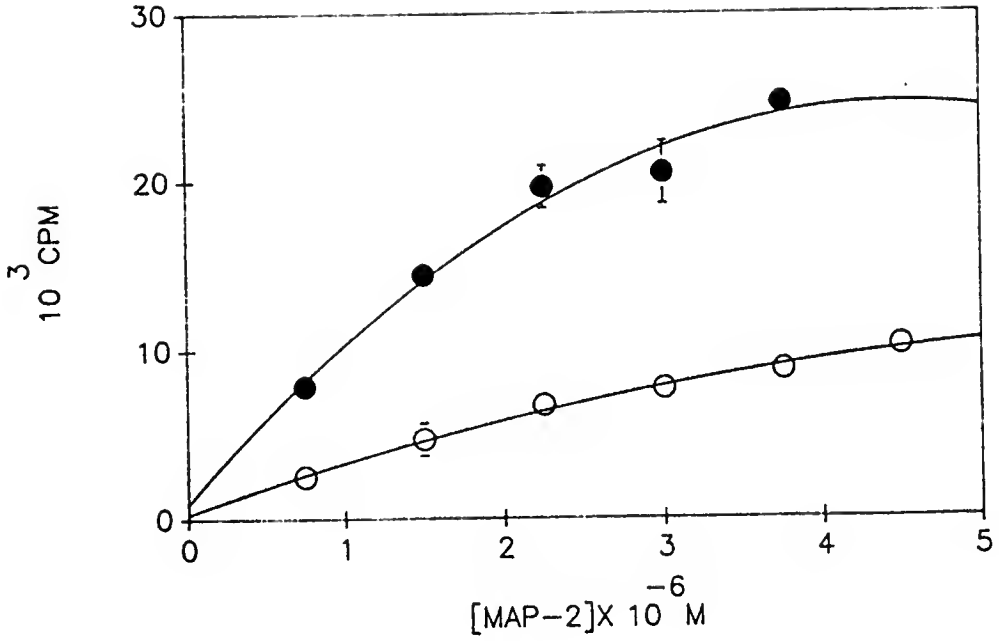


Fig. 4-14 Radiolabeled MAP-2 binding to taxol-stabilized microtubules in the presence and absence of 1.5 mM m_2' peptide. Plot of bound MAP-2 vesus total MAP-2 in absence (closed circles) and presence (open circles) of m_2'. In this experiment the microtubules were diluted into a solution containing radiolabeled MAP-2 with or without 1.5 mM peptide.

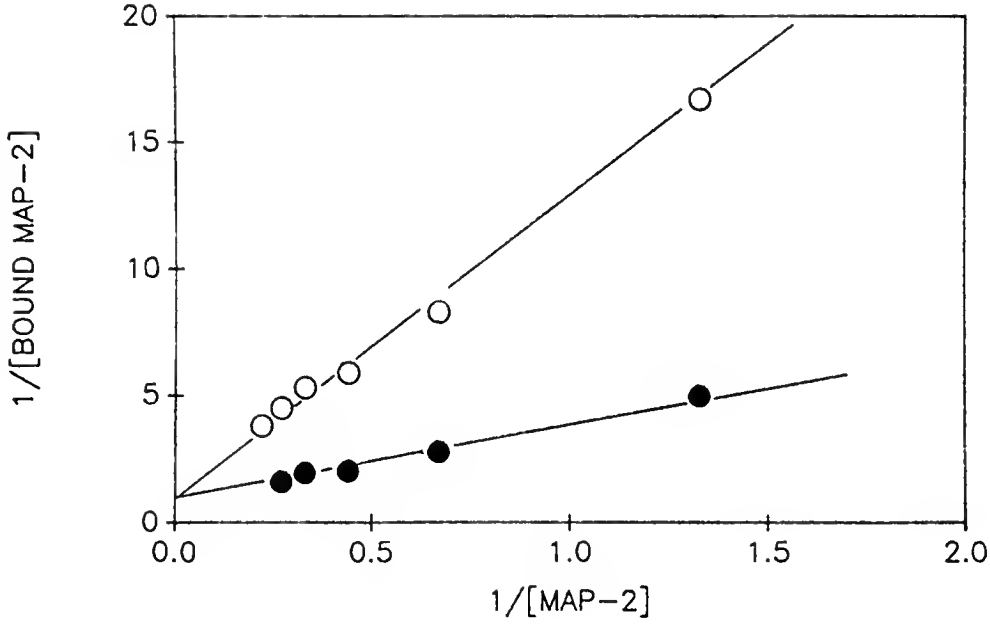


Fig. 4-15 Double reciprocal plot of MAP-2 binding. The data from Fig. 4-14 were transformed into reciprocals and plotted. With peptide (open circles), without peptide (closed circles).

change in slope, the m_2' inhibition constant was estimated to be 0.5 mM. Thus, the dissociation constant for MAP-2 is about 100 times less than the corresponding constant for a peptide-m_2.

Discussion

The experiments described in this chapter were designed to gain further insight about the microtubule-binding fragment of MAP-2 and determined what sequences were responsible for MAP-2 binding to microtubules. I have demonstrated that a single octadecapeptide corresponding to the second repeated sequence (from Val-1705 through Gly-1722 in murine MAP-2) promoted microtubule nucleation and elongation. To my knowledge, these are the first observations that a sequence amounting to less than 1% of the overall MAP-2 molecule is sufficient to interact with tubulin, but some additional considerations of MAP-2 structure seem appropriate.

That the second repeated sequence in murine MAP-2 can promote microtubule assembly is indeed interesting. Lewis et al. (1988) had studied microtubule binding of an *in vitro* translation product spanning amino acids 1621 through 1722 (including the first and second repeated sequences). While they did not attempt to demonstrate promotion of microtubule assembly, Lewis et al. (1988) succeeded in showing that their radioactively labeled 100-residue polypeptide copurified through two cycles of assembly/disassembly with MAP-containing microtubule protein. I found that a single octadecapeptide can not only copurify with tubulin, but can

promote tubulin polymerization at concentrations below the critical concentration of pure tubulin in vitro, even in the absence of MAPs.

While the possibility of multiple binding of peptide m_2 to tubulin cannot be discounted, establishing the stoichiometry of ligand binding to a protein can be particularly challenging for ligands that bind in the millimolar concentration range. This is due to the fact that most techniques are not sensitive enough to accurately measure the protein-ligand complex versus the free ligand concentration with dissociation constants in the millimolar range. It should also be emphasized that approximately 0.5 mM of the peptide m_2 is needed to promote assembly, but MAP-2 is effective in the 1-5 μM range. This may mean that the several repeats in MAP-2 reinforce each other in promoting assembly or that the octadecapeptide cannot readily assume the assembly-promoting conformation. Obviously, the conformational latitude of the second repeated sequence could be greatly influenced by the other residues in the entire microtubule-binding domain of MAP-2. At this point, however, the combined findings of Lewis et al. (1988) and these observations attest to the importance of the second octadecapeptide in MAP-2 interactions with tubulin.

It was interesting to note that just three additional amino acids at the carboxyl terminus of m_2, yielding m_2', promoted tubulin polymerization at lower peptide concentrations. This could have been due to the increased positive charge by adding a lysine and arginine interacting

with the anionic carboxyl termini of alpha and beta tubulin, or it may have been due to a more restricted conformation at the tail of the peptide that promoted tubulin polymerization.

The peptide corresponding to the second repeated sequence has an overall isoelectric point that is more basic than the first and third octadecapeptides (e.g., m_1, m_2 and m_3 have calculated pI values of 10.5, 11.6, and 8.0). In the pH 6.8 assembly buffer, m_1, m_2 and m_3 have overall charges of +3, +4, and +0.4, respectively. There are other indications that ionic interactions are important in MAP-2 binding to tubulin and/or microtubules. Flynn et al. (1987), for example, showed that the 28 kDa tubule-binding fragment of MAP-2 had an isoelectric point of about 10.5 whereas the larger projection arm fragment was considerably more anionic (pI about 4.8). Earlier findings suggested that MAP-2 interacts with microtubules largely through ionic forces (Vallee, 1982). Aside from the ability of intermediate salt concentrations (e.g., 0.4 - 0.6 M NaCl) to block MAP binding to tubules, the inhibitory action of polyanions and polycations should also be noted. Among the polyanionic inhibitors of assembly are RNA and polyglutamate (Bryan, 1976; Bryan et al., 1975), phosphatidyl inositol (Yamauchi and Purich, 1987), and estramustine phosphate (Wallin et al., 1985). These agents are thought to bind to the microtubule-binding region of MAP-2. Polycations also bind to microtubules and displace MAPs (Purich and Kristofferson, 1984). MAP binding is thought to occur at

the glutamate-rich C-termini of the tubulin α and ß chains, and the polycations presumably block these MAP-binding sites on tubulin and/or microtubules. Indeed, subtilisin treatment of tubulin results in loss of a 4 kD fragment containing the C-terminus, and tubulin proteolyzed in this manner readily assembles but fails to bind MAPs (Serrano et al., 1984). Nonetheless, I cannot conclude that ionic interactions alone determine the effectiveness of peptides in promoting assembly. An interesting case was m_1 and its analogue utilized in this chapter. The original repeat contained a lysine toward the carboxyl terminus and the analogue contained a glycine, but neither promoted microtubule polymerization. Obviously something else besides charge interactions was involved in proper binding to the microtubule-lattice, possibly a hydrophobic effect.

The observation that an octadecapeptide can promote assembly suggests a route for preparing low-molecular-weight modulators of microtubule assembly. As more information on the binding of MAP-2 to tubulin is developed, it may be possible to improve the binding efficiency of the oligopeptides. Already it has been shown that a slightly longer analogue, m_2', was a more efficient stimulator of microtubule polymerization. Moreover, the availability of these peptides should also permit additional studies of tau interactions with tubulin and MAP-2 interactions with other cytoskeletal elements such as the neurofilament proteins.

That MAP-1 and MAP-2 were both displaced from microtubules in the presence of peptides based on the second

repeated sequence suggested that peptides m_2 and m_2' can bind at or near the site(s) of MAPs interaction with microtubules. Until a quantitative binding assay for MAP-1 is developed, I cannot discern whether these peptides act as competitive inhibitors, as was found to be the case for MAP-2. At the same time, I can be confident that peptides m_2 and m_2' will be found to competitively inhibit MAP-2c binding to microtubules. This assumption is based on the interesting finding of Papandrikopoulo et al. (1989) that the embryonic MAP-2c has an identical amino acid sequence in the C-terminal microtubule-binding motif as the adult forms, MAP-2a and MAP-2b. The embryonic protein lacks 1372 amino acid residues spanning positions 147 to 1519 of adult rat MAP-2 as a result of an alternative splicing event.

That peptides corresponding to the second repeated sequence of MAP-2 can both promote assembly and displace MAPs opens the way for developing high affinity peptide analogues. I now have developed functional assays for the assembly-promoting and MAP-displacing characteristics of such peptides, and the latter can be extended by the electrophoresis experiments to test for specificity of MAP displacement (i.e., preferential desorption of a particular MAP). Again it has already been found that the extended second sequence peptide-m_2' was more effective than peptide-m_2 in displacing MAPs.

Finally, there is mounting evidence that a group of microtubule-associated proteins achieve binding to microtubules by way of a triad of nonidentical repeated

octadecapeptide sequences (Lewis et al., 1988). The stimulation of tubulin polymerization by synthetic peptides corresponding to several repeated sequences is consistent with, but does not prove, this hypothesis. On the other hand, the findings presented here indicate that peptides resembling the second repeated sequence of MAP-2 can displace MAP-2 from microtubules and can competitively inhibit MAP-2 binding. Because only these peptides promote tubulin polymerization, I am drawn to the conclusion that these peptides do indeed mimic MAP binding to microtubules.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

The experiments designed in this project focused on the microtubule-binding domain of MAP-2 with the aim of determining the sequences and/or regions responsible for several known functions and properties of MAP-2. During the course of these studies, I gained information on the structural features of MAP-2, its association with neurofilaments, and sequences responsible for promotion of both tubulin polymerization and MAP displacement.

Interactions with Neurofilaments

When MAP-2 is incubated with neurofilaments, the protein binds to the filaments and cosediments with them in a manner analogous to MAP association, or binding, with microtubules. The purpose of this research was to determine if the same region of MAP-2 that bound to microtubules was responsible for binding to neurofilaments. In part, this effort also addressed the question of whether a single MAP-2 molecule could cross-link a microtubule and neurofilament at the same time. If this was the case, MAP-2 could be responsible for many physiologically relevant connections microscopically observed within the neuronal cytoskeleton. Through the use of protease digests and ultracentrifugations of polymerized protein, the 28 kDa fragment of MAP-2 was shown to bind to both types of structures. This result

implied that a single MAP-2 molecule does not cross-link a microtubule and neurofilament at the same time; however, this does not exclude the possibility that MAP-2 is involved in connections between the two structures. If a higher order of MAP-2 structure such as a dimer exists, then two molecules of MAP-2 could cross-link the structures. To date no such dimer has ever been reported; only the 280 kDa monomer and a very large-molecular-weight aggregate composed of many MAP-2 molecules has been observed (Hernandez et al., 1986). Another possibility is that in vivo MAP-2 cross-linking is lost upon in vitro purification, perhaps the consequence of structural damage to the protein or the loss of another component necessary for cross-linking. While beyond the scope of this work, assays for cross-linking ability using falling-ball viscometry could be helpful. The addition of MAPs to microtubules, composed solely of tubulin, and neurofilaments causes a rise in the viscosity of the solution, but the addition of heat-treated MAPs or pure MAP-2 does not (Flynn and Purich, 1987). If MAP-2 is involved in the cross-linking of microtubules with neurofilaments, one could fractionate phosphocellulose-purified MAPs and add back various fractions to MAP-2 in the presence of microtubules and neurofilaments. The viscosity of the resulting solution can be monitored to check for cross-linking. With further fractionation it may be possible to identify key proteins in a complex that cross-links the two structures.

The identification of the 28 kDa microtubule-binding domain possessing a neurofilament binding site was never further analyzed for specific sequence interactions as was the case for microtubules. This was partly due to the lack of a functional assay for neurofilaments. Tubulin polymerizes in the presence of MAP-2 peptides but neurofilaments are already polymers and remain rather static in vitro. The peptides used in my work are probably too short to cross-link neurofilaments in a falling-ball viscometry assay. If the peptides were radiolabeled during synthesis (by using tritiated amino acids), one might be able to assay for cosedimentation of peptides with neurofilaments using liquid scintillation counting of the pellet and supernatant fractions. Alternatively, the peptides could be labeled after synthesis by acetylation of amino residues with ^{14}C-labeled acetate. A major pitfall of this technique is that the covalent modification of the peptide could alter its properties for binding to neurofilaments.

The binding of neurofilaments to MAP-2 was examined in a preliminary manner from a different approach to learn what part of NF-L binds to MAP-2. It had already been established that the L subunit of neurofilaments interacted with MAP-2 (Heimann et al., 1986). The L subunit sequence was known to contain one tryptophan residue in the middle of the rod forming domain common to all intermediate filament proteins. This tryptophan can be cleaved with BNPS-skatole to yield a 40 kDa carboxyl terminal fragment and a 30 kDa

amino terminal fragment. These fragments no longer form filaments; therefore cosedimentation is not a possible assay. Instead, the fragments were separated by SDS electrophoresis and electroblotted to nitrocellulose. The membrane was incubated with ^{32}P-labeled MAP-2 and exposed to X-ray film to determine which bands interacted with the MAP-2. Care must be taken in blocking the membrane after electroblotting and before incubating with the labeled protein. One has to include 2.5% (v/v) Triton X-100 with 10 mg/ml BSA in the blocking step. The BSA bound to any nitrocellulose not used in the electroblotting process and the added detergent displaced SDS from the blotted proteins. If SDS was not displaced, then the labeled MAP-2 bound everywhere on the blot and the autoradiogram appeared completely black. Removing the SDS allowed for a clear exposure. When ^{32}P-MAP-2 was incubated with a blot of skatole-cleaved NF-L, the labeled protein bound only to the 30 kDa amino terminus and intact, uncleaved NF-L. No interaction with the 40 kDa carboxyl terminal fragment was seen. An interesting note about use of this technique referred to specificity. While no interaction with the 40 kDa fragment was seen, several protein molecular weight markers showed weak interacions that could never be selectively removed with various salts or detergents. The most notable marker showing such an interaction was carbonic anhydrase. This line of research could be advanced with further cleavages of the 30 kDa fragment, but the non-specific binding to molecular weight markers should be

characterized and taken into consideration when analyzing binding to fragments of NF-L.

Structure of MAP-2

Valuable information on the overall organization of the molecule was gained through the use of protease digests and microsequencing techniques. The microtubule-binding fragment was derived from the carboxyl portion of the molecule and the projection domain from the amino portion. This was later confirmed by the cDNA work of Lewis et al. (1988). For such a large structure to yield only two distinct fragments after a thrombin digest would indicate some order of secondary structure; yet previous studies of MAP-2 by circular dichroic measurements indicated that MAP-2 possessed little organized secondary structure (Hernandez et al., 1986). Also, the cDNA-derived amino acid sequence revealed little secondary structure upon computer-assisted structural analysis. If there was little organized structure, one would expect far more than the two major fragments in thrombin digests, especially with eleven arginine residues present in the 28 kDa binding domain alone and many more present in the larger projection domain.

When MAP-2 was analyzed by electron microscopy, the structure most often seen was an elongated rod with a globular head at one end (Voter and Erickson, 1984). It is tempting to speculate that the globular head is the microtubule-binding domain and that the protease-sensitive hinge region connects the globular head and the elongated tail. The low level of observed secondary structure in the

whole MAP-2 molecule does not exclude the possibility that a small portion of the protein has organized secondary structure. Indeed, the microtubule-binding domain is only one-tenth of the entire protein, and its secondary structure organization may be masked in circular dichroic measurements using the whole protein. Accordingly, circular dichroic measurements of the 28 kDa fragment could be carried out to check for local areas of secondary structure. The immediate carboxyl terminus of MAP-2 shares some homology with leucine zipper DNA-binding proteins implying that some helical structure is present (Lewis et al., 1989).

Another interesting avenue to pursue regarding MAP-2 structure concerns the rat embryonic form, MAP-2c. This protein results from alternative mRNA splicing that leads to a deletion of residues 147 through 1519 (see Fig. 1-4). The protein contains the thrombin cleavage site of adult MAP-2 as well as the triad of octadecapeptide repeats and the amino terminal site for the regulatory subunit of cAMP-dependent protein kinase. It would be of interest to digest MAP-2c with thrombin to test if the same protease accessible site still exists. If the conformation in this hinge region is similar to the adult form, then a stable 28 kDa microtubule-binding domain could be produced.

MAP-2 Sequence Interactions with Microtubules

That the second octadecapeptide repeat of MAP-2 could promote tubulin polymerization *in vitro* was very interesting. At the time it was the first result indicating small sequences of MAPs could direct tubule assembly. A few

months later, the report of Ennulat et al. (1989) revealed that both the first and second octadecapeptide repeats of tau could promote tubulin polymerization. These sequences are similar to the MAP-2 repeated sequences, but the first repeat of MAP-2 could not promote tubulin polymerization. The third repeated sequences of tau and MAP-2 are similar in structure, and less basic than m_1, t_1, m_2, or t_2. Neither m_3 nor t_3 promotes microtubule formation. Nonetheless, a sequence that fails to promote tubulin polymerization may still interact with microtubules. These sequences could bind weakly to the microtubule lattice, and in MAP-2 they would be in close proximity to the microtubule wall already since m_2 interacts with tubulin. It is possible that m_2 directs the initial MAP-2 interaction with tubulin and that the m_1 and m_3 repeats bind afterward.

A test of binding for the m_1 and m_3 repeats to tubulin could be accomplished if the peptides are radiolabeled. One could then use taxol-stabilized microtubules composed solely of tubulin in a pelleting assay followed by liquid scintillation counting to check for cosedimentation of the peptides. An interesting variation of this experiment could be carried out to compare binding of the peptides to a microtubule lattice <u>versus</u> tubulin monomer. If the incubation of peptide with tubulin is done at 4°C and without taxol, the binding can be monitored by gel filtration chromatography. If the peptides bind, then a shift should be seen in their elution time from a gel filtration column. The peptide would coelute with the

tubulin monomer at approximately 100 kDa rather than at approximately 2 kDa without the presence of tubulin. Alternatively, one could use extrinsic fluorescence as a tool to monitor peptide interactions with microtubules. The peptides can be covalently modified with an extrinsic fluorochrome such as dansyl chloride and the emission spectrum can be monitored in the presence and absence of microtubules to observe possible changes. One caveat to this approach is the possible change in peptide properties after modification with the fluor. In that case, quenching or enhancement of the intrinsic tubulin fluorescence might prove to be useful.

To ensure that the peptides, including m_2, bind to the known site on tubulin, gel filtration again could be employed. It has been well established that the glutamate-rich carboxyl termini of both alpha and beta tubulin is the MAP-binding site (Serrano et al., 1984; Littauer et al., 1986). It appears that the second repeated sequence of MAP-2 binds at the same tubulin site as MAP-2 since the binding of the peptide is competitive with MAP-2 (see Fig. 4-15). To confirm this result the carboxyl terminal regions of both alpha and beta tubulin could be synthesized as was done for the MAP-2 peptides. Then these tubulin peptides could be incubated with the MAP-2 peptides to check for binding. If there is binding of the two sets of peptides, then a shift to an earlier elution time should be seen with a gel filtration column.

The MAP displacement studies demonstrated that the second repeated sequence of MAP-2 could displace MAP-1a and MAP-1b as well as MAP-2. This result suggests that MAP-1 binds to the same site or a nearby overlapping site. The microtubule-binding sequence for MAP-1 is a portion of the protein containing many KKEE and KKE(I/V) (Noble et al., 1989), indicating it has a different type of sequence interacting with microtubules than MAP-2. This sequence may interact with the carboxyl termini of alpha and beta tubulin at the same residues as MAP-2 or it could be slightly displaced. Another high-molecular-weight MAP from HeLa cells, designated MAP-4, contains 23 tandem repeats of KDMXLPXETEVALA (J. Olmsted, personal communication). It now appears there may be several distinct classes of MAP sequences responsible for microtubule binding and/or tubulin polymerization. The MAP-2- and tau-like sequences are listed in Fig. 6-1. Alignment of these repeats reveals several interesting features, including the conservation of the four carboxyl terminal residues and the spacing of positive charges, as discussed in Chapter 4. It is possible that peptide synthesis can be used to study the effects of potentially key residues on tubulin polymerization. Also, chemical modification of important residues may alter the ability of a peptide to promote tubulin polymerization. For example, cysteinyl thiol present in both MAP-2 and tau second repeats could be carboxymethylated to introduce a negative charge adjacent to the conserved positive charge at position four. Alternatively, the cysteine could be

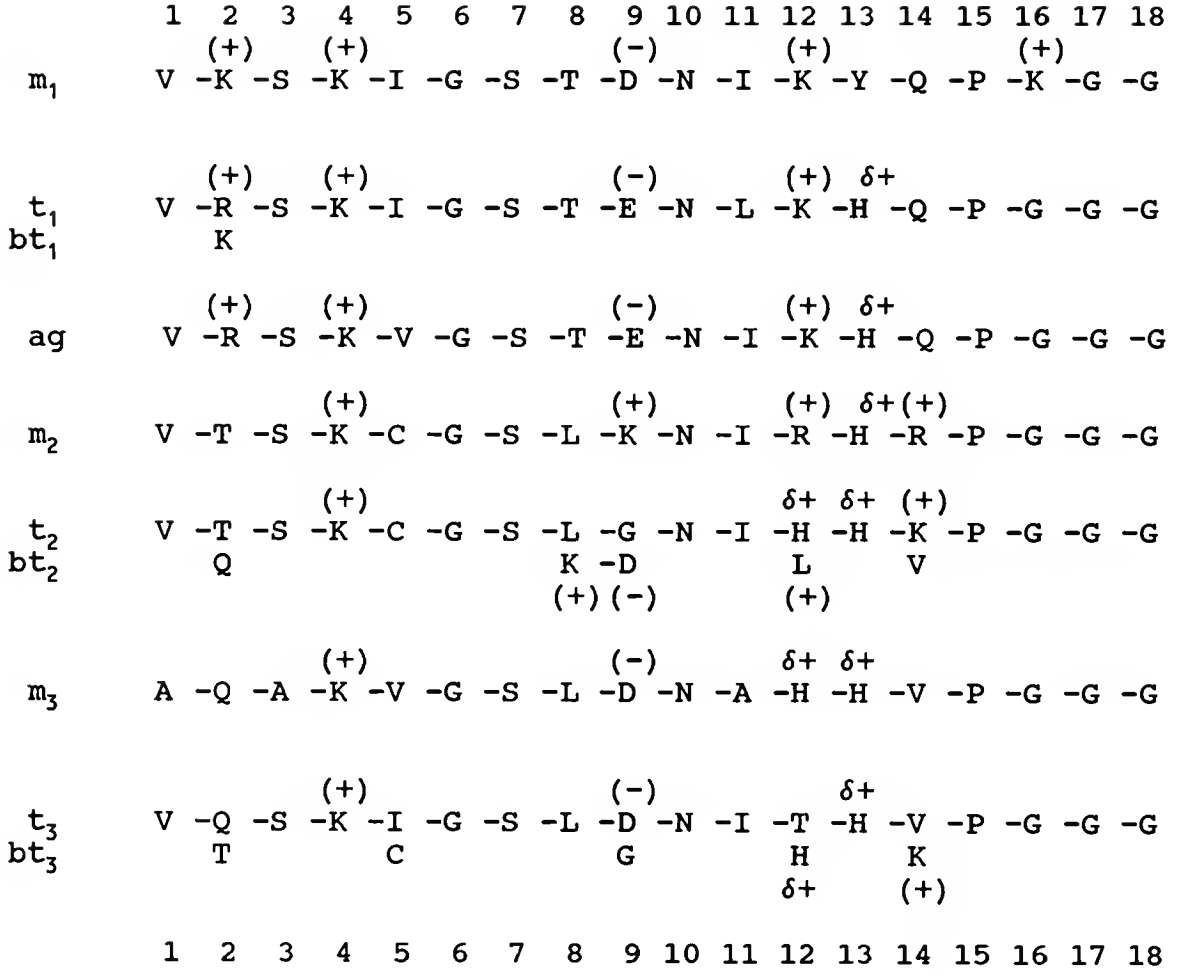


Fig. 5-1 The octadecapeptide repeats of murine MAP-2 (m_1, m_2, m_3), murine tau (t_1, t_2, t_3), bovine tau (bt_1, bt_2, bt_3), and a corresponding sequence of the 190 kDa adrenal gland (ag) are shown. The (+) signs represent full positive charges at pH 6.8, whereas the delta represents partially positive imidazolium side-chain groups of histidyl residues.

aminoethylated to increase the positive charge in this region. There is a wealth of potential work in determining essential residues for tubulin polymerization in the m_2 peptide, and converting a non-functional peptide such as m_1 to a polymerization promoting peptide.

The exact roles of the various microtubule-associated proteins in neurons remain to be elucidated. Nonetheless, the structural findings presented in this dissertation should be helpful in developing better probes of the <u>in vivo</u> interactions of MAPs with the neuronal cytoskeleton.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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